INVESTIGATION OF ANTIOXIDANT DEFENSE PROFILES AND STRESS RESPONSES IN HAWAIIAN *POCILLOPORA DAMICORNIS*

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Dedication

This body of work is dedicated to my loving husband and parents. Your support and encouragement have helped remind me of why I love marine biology and, overall, have made me into a better person. To my siblings, Kai, Joey, and Kei; thank you for keeping me grounded and for the humor that brings hearty laughs to the soundtrack of my life. To my extended family, both in Hawaiʻi and on the Mainland – especially my Aunties, from attending my wind ensemble concerts to supporting me during my defense, you have helped cement my sense of belonging in these islands while I conducted my research. I will never take that for granted. And, to Tucker and Bo, "woof". Thank you for trading never-ending pets for console when I just needed ears to listen and wagging tails to affirm that things will go right. Mahalo palena ʻole.

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I also want to acknowledge the place traditional Hawaiian ‘oli, or chants, have in shaping my path through this degree and their integration into my collection protocol. By chanting “E Hō Mai” and “Nā ‘Aumākua” prior to each collection, I sought to ask for knowledge and permission to enter the collection site, and then give thanks for the coral taken with “‘Oli Mahalo” to signify the end of sampling.

General Abstract

In order to improve development and refinement of molecular biomarkers for the evaluation of sub-lethal levels of stress in corals, this research provides a comprehensive characterization of baseline cycling of oxidative stress defenses in the coral *Pocillopora damicornis* and their responses to a natural cycle of thermally-induced bleaching. Such antioxidant enzymes are highly valuable as biomarkers for detecting stress under a wide breadth of stressors, and with global degradation of coral reefs occurring due to increasing instances of stressors implicated in oxidative stress, investigation of these biomarkers for specific tailoring towards coral health evaluations is critical. Further, through the investigation of these enzymatic responses to thermal stress, we aim to provide insight into oxidative stress responses of *Pocillipora damicornis* to thermal stress, which is tied to oxidative stress in corals and has become prominent, causing high levels of global coral bleaching and mortality, within the last decade. This work investigated cycling of antioxidant enzymes during coral reproductive cycles, responses of these enzymes to a thermal stress event resulting in mass coral bleaching, and whether *P. damicornis* preferentially shifts symbiont clade mutualisms to increase thermal tolerance following thermally-induced bleaching. Resultant from these data, this research suggests that reproduction has a significant effect on the activity of catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase. Further, enzymatic activity assays demonstrated significant increases in catalase activity with relation to heat-induced coral bleaching, although all other enzymes experienced no significant activity shifts. Finally, findings analyzing the potential for *P. damicornis* to shift symbiont clade assemblages from pre- to post-bleaching cycles suggests that this coral species favors conserving existing mutualisms. Such conclusions aid the molecular coral biology community through refining existing tools for detecting and characterizing sub-lethal stress levels in corals.

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List of Abbreviations

CAT	Catalase
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
GR	Glutathione reductase
°C	Degrees Celsius
ROS	Reactive oxygen species
KML	Kewalo Marine Laboratory
S9	Post-mitochondrial protein fraction isolate
DNA	Deoxyribonucleic acid
kB	Kilobase
kDa	Kilodalton
Se	Selenium
CHP	Cumene hydroperoxide
PMSF	Phenylmethylsufonyl fluoride
mg	Milligrams
µg	Micrograms
mL	Milliliters
µL	Microliters
mM	Millimolar
µM	Micromolar
CI	Confidence interval
SD	Standard deviation

Chapter 1: General Introduction

In Hawaii alone, coral reefs are estimated to contribute approximately $364 million in tourism revenue, fishery catch, and coastal erosion and storm protection (Cesar & Van Beukering, 2004). These same services are worth roughly $800 billion globally, and are increasing in value (Cesar, Burke, & Pet-soede, 2003). Furthermore, estimations suggest coral reefs provide over 10% of the global fish stock for human consumption (Moberg & Folke, 1999). Their high value to global food production, tourism, coastline protection, and ecosystem health/diversity cast these habitats as foundations for economic and environmental health (Bishop et al., 2011; Brander et al., 2007). Despite their critical role, increased anthropogenic impacts and changing abiotic environmental parameters are destroying coral reefs around the globe (Downs et al., 2012; Giordano et al., 2007; Mydlarz & Palmer, 2011). Blast fishing, terrestrial runoff and pollution, sewage effluent infusion, increasing coral disease, intensifying global storms and invasive species introductions over the last three decades have resulted in major losses in coral cover, associated fish abundance, and decreased ability of coastal habitats to support local human populations (Figure 1) (Bruno, Petes, Harvell, & Hettinger, 2003; Cesar et al., 2003; Dailer, Knox, Smith, Napier, & Smith, 2010; Fabricius, 2005, 2011; Fox, Pet, Dahuri, & Caldwell, 2003; Giordano et al., 2007; Kaniewska et al., 2012; Stimson, Larned, & Conklin, 2001).

Figure 1. Sedimentation, overfishing, invasive algal overgrowth, and rising sea temperatures all threaten the health of coral reefs (photo credit: Maui Tomorrow Foundation, NOAA Fisheries, Chris Runyon, and Duke University).

Nutrient enrichment, higher temperatures, and oxidative stress are considered the leading factors contributing to the increase in the incidence of coral disease, bleaching, and death (Bruno et al., 2007, 2003; Desalvo et al., 2008; Fabricius, 2005; Harvell et al., 1999; Lesser, 1997; Mapstone & Fowler, 1988; Nagelkerken et al., 2000; Stone, Huppert, Rajagopalan, Bhasin, & Loya, 1999; van de Water, Lamb, Heron, van Oppen, & Willis, 2016; Ward, Kim, & Harvell, 2007). However, the impact of these factors on biochemical pathways and methods to detect such impacts in corals need comprehensive characterization, such that faster and more accurate measures of stress can be conducted prior to coral death (Downs et al., 2002; Downs, Mueller, Phillips, Fauth, & Woodley, 2000; Mydlarz & Palmer, 2011; C. V. Palmer & Traylor-Knowles, 2012; Caroline V. Palmer, Bythell, & Willis, 2012; J. R. Ward & Lafferty, 2004). Through combined early stress detection and stress prevention, there is potential to combat changing environmental parameters that threaten reefs globally.

Oxidative stress is of particular interest in coral research due to increasing incidences of stressors that lead to oxidative damage, such as high irradiance, hypoxia, hyperoxia, ocean acidification, sedimentation, algal overgrowth interactions, and xenobiotic exposure, all of which are of significant importance within waters throughout

the Hawaiian archipelago (Bruno et al., 2007; Fabricius, 2005; Higuchi, Fujimura, Arakaki, & Oomori, 2008; Lesser, 1997; Lesser, Stochaj, Tapley, & Shick, 1990; Martinez, Smith, & Richmond, 2012; Rands, Douglas, Loughman, & Ratcliffe, 1992). The response of corals to oxidative stress can manifest as expulsion of zooxanthellae resulting in bleaching, tissue loss, decreased metabolic activity, decreased aerobic respiration, increased disease susceptibility, and eventually mortality (Brown, 1997; Bruno et al., 2007; Fabricius, 2005; Higuchi et al., 2008; Lesser et al., 1990; Lesser, 2006; Palmer & Traylor-Knowles, 2012; Palmer, Bythell, & Willis, 2010; Rands et al., 1992; Rougée et al., 2006). To determine when a coral is under oxidative stress, prior to drastic physical manifestations, an understanding of the molecular indicators using diagnostic biomarkers must first be achieved.

In normal metabolic processes, reactive oxygen species (ROS), such as hydrogen peroxide and oxygen radicals are produced during photosynthesis and aerobic respiration (Devasagayam et al., 2004). Further, these reactive oxygen species are known to aid in homeostatic functions, such as apoptosis, neurotransmitter release, and immune system reactions (Lesser, 2006). However, the effects of oxidative stress and cellular damage by ROS occur when ROS production is greater than the antioxidant enzyme capacity for elimination, leading to the accumulation of ROS within the cell (Bellantuono, Granados-Cifuentes, Miller, Hoegh-Guldberg, & Rodriguez-Lanetty, 2012; Devasagayam et al., 2004; Richier et al., 2003; Ulstrup, Hill, & Ralph, 2005).

A transition from natural ROS production under normal physiological conditions to pathological proliferation can result in indiscriminate binding to DNA, RNA, lipids, and proteins, oxidizing these molecules, resulting in 'oxidative damage' (Devasagayam et al., 2004; Kaniewska et al., 2012). Ionizing radiation, heat, inflammation, and overexposure to metals and other xenobiotics can induce reduction of oxygen (O_2) or oxidation of water (H_2O) into ROS, such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) (Devasagayam et al., 2004). Damage caused by lipid peroxidation can lead to disruptions in the lipid bilayer of cells and premature cell death, while oxidation of proteins can lead to the inactivation of key enzymes and damage to organs implicated in detoxification of tissues (e.g. liver), and, most disruptively, oxidation of DNA can lead to potentially oncogenic mutations (Lesser, 2006; Waris &

Ahsan, 2006). These effects not only threaten cellular function, but may also leave affected organisms more vulnerable to disease, and in the case of corals, can instigate bleaching (Downs et al., 2002; Lesser, 1997).

Antioxidant enzymes help mitigate the effects of ROS by converting them to less reactive or non-reactive molecules, such as oxygen and water (Bellantuono, Granados-Cifuentes, Miller, Hoegh-Guldberg, & Rodriguez-Lanetty, 2012; Giordano et al., 2007; Mansour & Mossa, 2009; Mapstone & Fowler, 1988; Palmer et al., 2011; Ross et al., 2000; Rougée, Downs, Richmond, & Ostrander, 2006). Of those included in the greater antioxidant defense network (figure 2), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) are extensively studied and well cataloged as having major roles in reducing major molecules of oxidative damage. Superoxide dismutase, for example, directly catalyzes the reduction of highly reactive superoxide anions to hydrogen peroxide and water, and several isozymes of SOD can be found in the cytosol (Cu/Zn SOD), the mitochondria (Mn SOD), and chloroplasts (Fe SOD) in animal and plant cells. Prevalent in most organisms and commonly localized in the peroxisome, CAT achieves further detoxification of intracellular ROS through the dismutation of hydrogen peroxide to oxygen and water. Concurrently, this reaction can be achieved through the activity of GPx and the oxidation of the antioxidant, reduced glutathione. This results in the reduction of hydrogen peroxide into water, shunting the now oxidized glutathione to GR, which functions to replenish reduced glutathione stores through the oxidation of molecules of nicotinamide adenine dinucleotide phosphate (NADPH). However, though studying the response of these enzymes is useful for the analysis of coral health and expansion of knowledge in this field of work, it also requires a knowledge of basal profiles of coral ROS response during homeostasis.

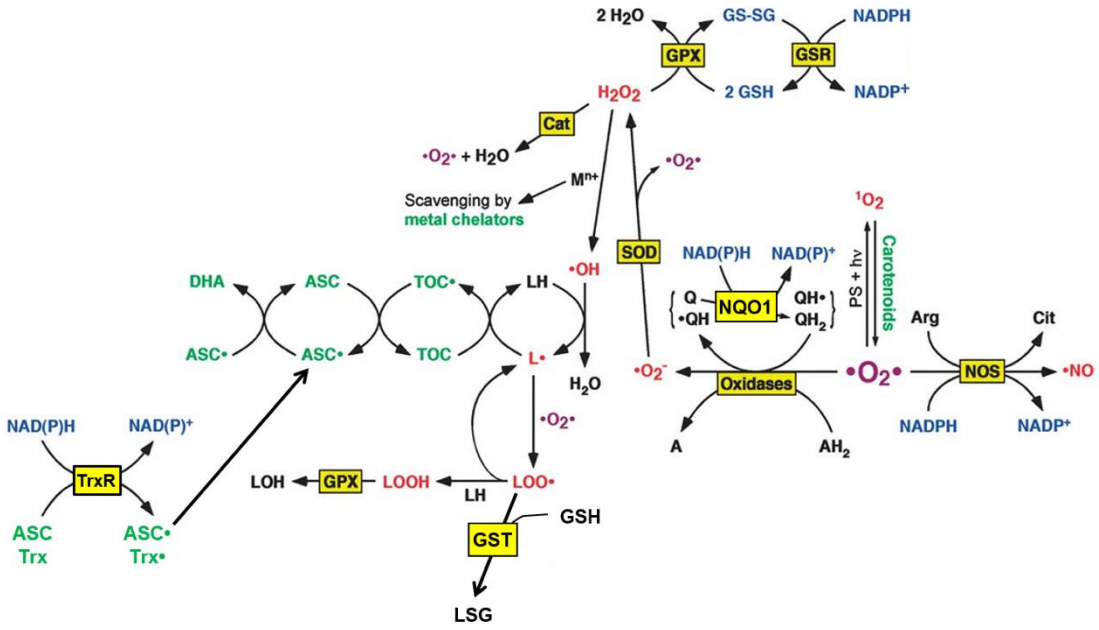


Figure.2. Antioxidant defense network schematic displays major pathways through which reactive oxygen species and other reactive molecules may be broken down into non-reactive, or less reactive forms (This diagram is a modification of the original figure from (Sorg, 2004)*).*

Although basal levels of stress can be accounted for by using experimental controls for specific exposure assays, these "background" stress loads have the potential to shift with time points and seasons (Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010) and even reproductive cycling (Rougée, Richmond, & Collier, 2014). Thus, the first step in developing and refining assays to analyze the expression response of enzymes, such as SOD, CAT, GPx, and GR to varied etiologies of oxidative stress is to first analyze the shift in the expression and activity of these enzymes over simple reproductive cycles. Yet, this can be difficult to accomplish, as many corals spawn or brood on yearly cycles, necessitating long-term studies of colonies for baseline stress development (Fadlallah, 1983; Harrison & Wallace, 1990). Instead, by employing the coral *Pocillopora damicornis* as a model for baseline antioxidant stress characterization, experimental timescales can be shortened to weekly surveys, focusing on their monthly brooding cycle (Richmond & Jokiel, 1984)(figure 3).

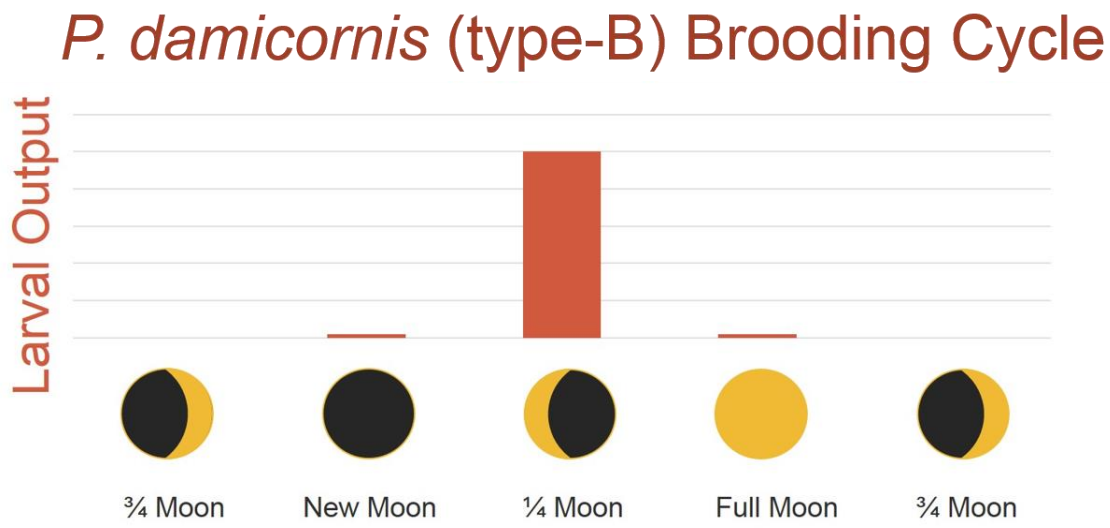


Figure 3. Pocillopora damicornis (type-b) brooding cycle with peak planula release at 1/4 moon phase.

Furthermore, expanding studies to the symbiotic zooxanthellae that function as the principal energy source for coral and main producer of ROS under high stress loads, such as thermal extremes, provides greater resolution of potential changes to holobiont health under oxidative stress. Such work aids in identifying if *Symbiodinium* recruitment may change during and following stress events as an adaptive response to stress events through the recruitment of stress-tolerant clades (Cunning, Ritson-Williams, & Gates, 2016; Silverstein, Cunning, & Baker, 2015). Research by Stat, Morris, & Gates (2008) found that of the known symbiotic dinoflagellate lineages (clade A-H), corals preferentially recruited clade C, which was found to facilitate relatively higher rates of primary productivity and coral growth through a greater ability to fix carbon, but has also been found to be less heat-tolerant (Cunning et al., 2016; Silverstein et al., 2015; Stat et al., 2008). Notably, clades, such as clade A, have been characterized to provide corals with less nutrients and fixed carbon, and have been proposed to hold a more parasitic role in corals mutualisms, capitalizing on recruitment during or post-bleaching and found in corals with significantly higher incidences of disease (Lesser, Stat, & Gates, 2013; Stat et al., 2008). Additional research has found corals to recruit clade D, a more thermally tolerant, but less energy productive clade of *Symbiodinium,* following thermal bleaching events, suggesting that under thermal stress, corals will sacrifice food production for bleaching security (Cunning et al., 2016; Silverstein, Cunning, & Baker, 2017). These

findings point towards important symbiont clade variations with potential impacts to future coral reef health as an area of interest for future research. Integrating simple examinations of whether coral symbiont recruitment changes with respect to heat stress promotes greater understanding of the adaptive, in addition to the enzymatic, capacity for corals to respond to stress.

Herein we have investigated, 1) the presence and activity of several antioxidant enzymes in the coral species *P. damicornis* during their reproductive cycle, 2) determined whether these pathways could be used as diagnostic biomarkers to indicate when corals are subjected to oxidative stress during heat exposure, and 3) identified whether *Symbiodinium* clade variations were present from pre-bleaching to bleaching to post-bleaching states in these corals.

Previous studies have identified increased expression of SOD, CAT, GPX, and GR in corals that underwent an oxidative stress event (increased temperature, hypoxia, hyperoxia, xenobiotic exposure) resulting in their bleaching (Downs et al., 2000; Higuchi et al., 2008; Lesser et al., 1990; Palmer et al., 2011; Richier et al., 2003; Richier, Furla, Plantivaux, Merle, & Allemand, 2005; Rougée et al., 2006; Van Oppen & Gates, 2006). Further, as a hermaphroditic species that undergoes a monthly reproductive brooding cycle (Richmond & Jokiel, 1984), multiple reproductive cycles can be analyzed in rapid succession, reducing variation in year-to-year a/biotic parameters that could affect sample quality. This is in comparison to other common Hawaiian species, such as *Montipora capitata*, another hermaphroditic coral that instead undergoes yearly reproductive spawning cycles, and *Porites* spp., a genus containing gonochoric corals that spawn on yearly cycles (Kolinski & Cox, 2003). By removing the influence of year-to-year environmental flux and differences in expression and activity of enzymes based on sex, we look to reduce error and improve the accuracy of our study.

With these choices in mind, identification of SOD, CAT, GPX, and GR activity in this study would be illustrative of innate levels of defensive enzyme presence and activity under normal environmental conditions with relation to reproductive cycling. Further insight will be gained as to how these antioxidant defense enzymes are implicated in stress response under coral bleaching. Inclusion of *Symbiodinium* clade variations in subject corals over a bleaching event will indicate potential basal variances in the ability

of certain individuals to withstand stress inputs, such as heat, and long-term intra-colony fitness variability.

By determining enzyme activity and background expression levels, this research can aid in the creation and utilization of diagnostic tools to measure coral stress on a molecular level, prior to physical coral damage from oxidative stress. Moreover, by selecting the enzymes SOD, CAT, GPX, and GR for study to better characterize this stress, efforts to elucidate which markers work best when diagnosing oxidative stress and the degrees to which enzyme response is upregulated can be better focused. This work will also expand the understanding of potential zooxanthellae clade shifts in corals and provide insight examining the molecular implications of bleaching and post-heat stress recovery stage. These data will help guide conservation efforts through the investigation and comparison of the effect of oxidative stressor exposure on coral health. Additionally, this project will help address issues of watershed degradation by adding these enzymatic activity assays, Western Immunoblot, and symbiont variability analyses to the toolbox of analytical skills and metrics of change. Results of this project will help to better inform experimental design and reduce time spent developing assays for future researchers studying coral health.

Chapter 2: LITERATURE REVIEW

Antioxidant Enzyme Cycling over Reproductive Lunar Cycles in *Pocillopora damicornis*

Corals play critically important roles in the structural and biological hierarchy, and health of coral reef environments (Birkeland, 1997). With greater levels of anthropogenic stress impacting global marine environmental health (Gattuso et al., 2015; Heron, Maynard, van Hooidonk, & Eakin, 2016; Hughes et al., 2017; Maynard et al., 2015), it has become increasingly important that techniques for evaluating coral stress prior to reef collapse are developed and applied (Edge, Shearer, Morgan, & Snell, 2013). It is no longer sufficient to simply track bleaching and mortality as primary indicators of stress in corals. As such, there have been recent advances in research investigating the application of molecular analyses to facilitate sub-lethal stress evaluations (Ainsworth, Hoegh-Guldberg, Heron, Skirving, & Leggat, 2008; Barshis, Ladner, Oliver, & Palumbi, 2014; Desalvo et al., 2008; Downs et al., 2012; Edge et al., 2013; Rougée et al., 2006). The development of new diagnostic tools and the further refinement of existing stress detection methods must be carried out to meet future demands for addressing coral stress to inform policy and improve conservation efforts.

One way that this can be accomplished is by developing tests for the evaluation of specific stress responses. Antioxidant stress enzymes, for example, are useful for the analysis of impacts on the health of coral animals due to their wide applications in response to a variety of stressors (Downs, Richmond, Mendiola, Rougée, & Ostrander, 2006; Higuchi, Yuyama, & Nakamura, 2015; Vijayavel, Downs, Ostrander, & Richmond, 2012). Understanding antioxidant enzyme presence and activity in coral tissues has the potential to be employed as a metric for evaluating stress in reefs, including gradients of stress, and pin-pointing the impacts of natural and xenobiotic toxicants on the health of corals (Edge et al., 2013; Rivest & Hofmann, 2014). These evaluations may aid in determining the degree of stress being conferred on specific areas of the reef by environmental perturbations, or the gradient over which a source point of pollution may be diffusing across a space (Downs et al., 2006). In the future, a vast library of biomarkers may prove valuable in aiding communities by rapidly calculating the damage of onshore impacts or lending managers the prescience to act on potential environmental stress prior to coral death.

Previous studies have highlighted antioxidant enzymes as critical biomarkers of the impacts of stressors such as heat, xenobiotic exposure, and high-irradiance (Downs et al., 2006; Higuchi et al., 2015; Liñán-Cabello, Flores-Ramírez, Cobo-Díaz, et al., 2010; Olsen, Ritson-Williams, Ochrietor, Paul, & Ross, 2013). However useful this suite of enzymes is in providing information about coral stress response and threat levels, many of the substrates that trigger this type of stress are naturally produced in normal homeostatic processes (Agarwal, Gupta, & Sikka, 2006; Dowling & Simmons, 2009; Fujii, Iuchi, & Okada, 2005). Due to the utility of adding these biomarkers to the host of tools that can be employed to evaluate reef health, it is important to know baseline levels of protein expression and activity, especially over varying time points and potential sample periods. Of course, many studies help counter any confounding factors generated by background levels of stressors through the implementation of controls. However, in order to have full confidence in using these enzymes as biomarkers for stress detection, it is important to take into consideration how endogenous levels of expression may change over shifting baselines. As such, prior to adopting these enzymes into our suite of diagnostic tools, we seek to characterize whether reproductive cycling has a discernable effect on the enzymatic profile of a major species of coral, *P. damicornis*.

Coral reproduction is a focus of this research, in part due to previous work describing cyclical variation in defense enzyme activity during reproduction events. A study performed by Rougée, Richmond, & Collier (2014) illustrated variations in the expression and activity of xenobiotic metabolizing enzymes during reproductive cycling in the coral *P. damicornis*. Glucuronosyltransferase, glutathione-s-transferase (GST), cytochrome P450 2E1, and cytochrome P450 reductase were all found to fluctuate significantly over natural reproductive lunar cycles (Rougée et al., 2014). Additionally, research by Ramos, Bastidas, Debrot, & García (2011) provided insight into the effect of reproductive cycling on various biotransformation and antioxidant enzyme activities. In their work, activities of cytochrome P450s, GST, NADPH c reductase, and catalase (CAT) were all significantly higher during reproductive peaks in the coral *Siderastrea siderea* (Ramos et al., 2011). With such evidence for the cycling of enzymatic activity tied to reproductive cycling, coupled with the knowledge of ROS impacts on the health of reproductive systems in other organisms, the lack of more comprehensive research into

antioxidant enzyme expression over reproductive cycles in corals underlines a hole in the foundation of antioxidant enzyme expression understanding (Agarwal et al., 2006).

As previously referenced, reproduction is an innate source of reactive oxygen species (ROS) generation and relies heavily upon the interplay of pro-oxidants and antioxidants (Agarwal et al., 2006; Fujii et al., 2005; Halliwell & Gutteridge, 2015; Rahal et al., 2014). This interplay of ROS production and detoxification during reproduction has a critical role in both aiding and inhibiting high quality gamete production, fertilization, and embryo development (Fujii et al., 2005). In systems other than those found in Cnidarians, studies have pointed to a heightened prevalence of ROS, potentially impacting fertility, as well as ROS being implicated in the termination of embryos and reproductive senescence due to heightened levels of oxidative stress (Agarwal, Gupta, & Sharma, 2005; Agarwal et al., 2006; Carbone et al., 2003). Oxidative stress also has the potential to reduce embryo growth and decrease fertilization rates (Agarwal et al., 2006). However, ROS have been found to have both beneficial and detrimental effects on the motility and viability of sperm cells. Specifically, sulfoxidation is required for the maturation of sperm and packaging of nuclei in sperm heads (Fujii et al., 2005). Excess ROS proliferation acting upon the axoneme of spermatozoa, however, can either depress or inhibit motility (de Lamirande & Gagnon, 1992). Rich in polyunsaturated fatty acids, spermatozoa are highly vulnerable to lipid peroxidation due to low cytosolic availability of ROS-scavenging enzymes (Agarwal et al., 2006; Saleh & Agarwal, 2002). As a result, low or unregulated levels of lipid peroxidation can lead to the production of spermicidal compounds, such as (E)-4-Hydroxy-2-nonenal, which at concentrations of only 50 µm, can result in irreversible motility loss (Selley, Lacey, Bartlett, Copeland, & Ardlie, 1991). Antioxidant compounds, like glutathione, and ROS-scavenging enzymes, such as superoxide dismutase (SOD), exist to modulate the effects of ROS on egg and sperm viability and promote embryo integrity (Agarwal et al., 2006).

Although corals may have different reproductive methods than vertebrates, other invertebrates, and plants, there are similar processes with respect to ROS generation and detoxification that are highly conserved across taxa and are required for optimizing reproductive integrity (Dowling & Simmons, 2009). To improve the breadth and quality of the biomarkers available for this work, this project sought to define basal enzymatic

stress levels in a major coral species of study, with broad global application, with respect to reproductive cycling (Hoeksema, Rodgers, & Quibilan, 2014). Further, unlike other common reef-building corals, such as various *Porites* spp., *Acropora* spp., and *Montipora* spp., that utilize external fertilization and planula larvae development through seasonal mass-broadcast spawning events following annual cycles (Harrison et al., 1984; Harrison & Wallace, 1990; Neves, 2000; Padilla-Gamiño & Gates, 2012; Stimson, 1978), the coral *P. damicornis* was chosen for study due to its monthly brooding cycles, with peak reproductive output closely tied to the ¼ moon phase (Richmond & Jokiel, 1984). As such, reproductive shifts in antioxidant enzyme activity were observed over monthly cycles, rather than being drawn-out over a year. This also reduced the potential for seasonal variations and year-to-year changes in environmental stressors to affect the comparison of reproduction against potential antioxidant enzyme activity cycling (Cooper, Gilmour, & Fabricius, 2009; Harrison & Wallace, 1990; Selina Ward, 1995). Through the improvement of baseline knowledge investigating potentially inherent stress levels in major reef-building corals, future studies examining coral health can better account for the degree of stress endured by corals with respect to natural phenomena. Furthermore, the study of such natural phenomena will help identify whether acute changes to coral enzyme profiles warrant greater consideration over reproductive peaks and troughs.

Potential Enzymatic Indicators of Coral Bleaching Induced by Heat Stress

Coral bleaching is the physical manifestation of a breakdown in the obligate relationship between host corals and their single-celled algal symbionts (*Symbiodinium* spp.), commonly referred to as zooxanthellae (Jokiel & Coles, 1977). This breakdown leads to the expulsion of *Symbiodinium* spp. from the host coral's tissues (Gates, Baghdasarian, & Muscatine, 1992), and is visually indicated by loss of coral tissue pigmentation, and causes a wide array of physiological consequences. Though bleaching has implications in stress reduction under extreme trauma, the degradation of the coral holobiont results in a loss of energy production for the coral host, and can lead to reproductive cycle disruption (Paxton, Baria, Weis, & Harii, 2015; S Ward, Harrison, & Hoegh-Guldberg, 2002), reduced survival of coral planula larvae (Schnitzler,

Hollingsworth, Krupp, & Weis, 2012), increased susceptibility to disease and other stressors (Bruno et al., 2007; Harvell et al., 1999; Maynard et al., 2015; Sudek, Williams, Runyon, Aeby, & Davy, 2015), and under prolonged bleaching periods, coral death (Bahr, Jokiel, & Rodgers, 2015; Hughes et al., 2017). While many factors such as disease, sedimentation, chemical exposure, salinity changes (too high or low), and algal competition/overgrowth can instigate a bleaching response, increasing frequency of heat-induced bleaching over the past three decades have caused mass-mortality of coral reefs globally, and will continue to be one of the biggest threats to future coral survival (Hughes et al., 2017).

Although corals are able to withstand mild levels of thermally-induced bleaching, chronic and severely warmer sea surface temperatures have led to massive coral die-offs (Baker, Glynn, & Riegl, 2008; Eakin et al., 2010; Wilkinson, 2004). The predictions for climate change forecast more severe and unpredictable storms, droughts, and sea level rise (Meehl et al., 2000; Storlazzi, Elias, & Berkowitz, 2015). Additionally, projections for rising sea surface temperatures predict that chronic thermal stress events will continue (Hughes et al., 2017). Such events resulted in mass coral mortality in the early 1980s, mid-90s, early 2000s, and past 4 years (Eakin et al., 2016; Heron et al., 2016; Hughes et al., 2017). Increases in coral stress events due to ocean warming are already occurring at a yearly pace, with bleaching extending throughout the Pacific during 2014-2016 and now chronically occurring throughout areas, such as the Hawaiian Archipelago from 2014 to present (Eakin et al., 2016). Heron, Maynard, van Hooidonk, & Eakin (2016) found that warming is occurring at almost all coral reefs in recent decades, where summer temperatures have been breaching previous records for the majority of reefs. Only one-third of reefs have been exposed to thermal levels that induce bleaching less than once per decade, while one-third of reefs have been exposed one to two times per decade (Heron et al., 2016). The remaining third of global coral reefs have experienced thermally-induced bleaching more than twice per decade. Furthermore, over the 28-year period surveyed, the number of global coral reef bleaching stress events tripled. However, bleaching stress has not been isolated only to the warmest months of the year. What was most worrying, were findings describing that one-quarter of all reefs included

in this study were found to be in a state of stress during times that did not coincide with the hottest months of the year (Heron et al., 2016).

Over this survey period, coral reefs warmed at a rate of about 0.22 C per decade, which varied with respect to region: the Middle East experienced the fastest rate of warming of approximately 0.32 C per decade versus that of the slowest warming region, Australia; at about 0.08 C per decade. Approximately 81% of global coral reefs have experienced thermal stress that had the potential to induce bleaching multiple times during the almost three-decade survey period. Further, instances of thermal stress could be found somewhere on the world's coral reefs every year from 1985-2015 (Heron et al., 2016). Current bleaching trends have surpassed thermal peaks from previous years, with 2014-on standing as the warmest years on record, causing mass coral bleaching for four consecutive seasons (Bahr et al., 2015; Eakin et al., 2016). Projections suggest that thermal stress-linked bleaching events will continue to increase in frequency and severity, with more than 98% of coral reefs expected to experience yearly bleaching events by 2050 (Van Hooidonk, Maynard, Manzello, & Planes, 2014; van Hooidonk, Maynard, & Planes, 2013).

If the scientific community can understand bleaching frequency, thermal increases, and potential areas of refuge from thermally-induced bleaching stress, this will aid in predictions of the severity of bleaching response and bleaching-induced mortality. Effectively, by conducting these studies we hope to provide essential biomarkers that can signal a bleaching (or other detrimental) event before it happens. This would allow the maximum possible conservation response. These data also provide conservationists with information on the most thermally resilient coral reefs and help increase the understanding of which reefs face the highest frequency of thermally extreme events, how frequency has changed over time and per season, and variability of warming over time (Eakin et al., 2016; Hughes et al., 2017). This study also highlights a serious issue in coral reef management. Namely, employing bleaching and mortality as metrics for evaluating coral health fails to address how stress is occurring on a molecular level (Ainsworth et al., 2008). With increasing prevalence of bleaching events, research has pivoted towards identifying molecular biomarkers of the coral stress response in order to better elucidate the biochemical and physiological mechanisms of coral responses to

heightened heat exposure (Downs et al., 2000; Gibbin, Putnam, Gates, Nitschke, & Davy, 2015; Merle, Sabourault, Richier, Allemand, & Furla, 2007; S Ward et al., 2002; Weston et al., 2015). The aim is to improve the scientific and marine conservation communities' understanding of how to best facilitate coral resilience and adaptation to increasing temperature stress and identify those coral reefs most at risk from temperatures that exceed the bleaching threshold.

Multiple investigators have explored identification of biomarkers for 'strong' coral genotypes that are thermally robust under extreme temperature pressure (Baker, 2014; Bellantuono et al., 2012; Mascarelli, 2014; Palumbi, Barshis, Traylor-Knowles, & Bay, 2014; Shinzato et al., 2011). In addition, Silverstein, Cunning, & Baker (2015) and Berkelmans & van Oppen (2006) have provided evidence of thermal resistance afforded by symbiont clade preference, which has the potential to increase survivorship and delay or decrease bleaching occurrence. Further, research by Downs et al. (2000, 2002) and Kenkel et al. (2011) sought to develop better molecular biomarkers for stress identification that can be employed to detect sub-lethal levels of stress, pre-bleaching, and provide profiles for those corals that may be best adapted in shifting their metabolic profiles to combat the products of increased thermal stress that lead to coral bleaching. Using these efforts as a base from which to continue expanding the breadth of knowledge in sub-cellular stress levels in corals under bleaching pressure, this study focused on evaluating the response of corals to the basal instigators of bleaching stress, reactive oxygen species (ROS).

The ROS are known for their potential for cytotoxicity (Rahal et al., 2014; Sorg, 2004). Examples include hydroxyl radicals and hydrogen peroxide, which can instigate apoptotic pathways through oxidation of lipids, DNA, RNA, and proteins, depressing or interrupting cellular processes and normal function (Sorg, 2004). Specifically within the coral holobiont, the work of Lesser (1997) suggests that increases in the presence of ROS are directly associated with the breakdown of coral-algal symbioses and subsequent expulsion of *Symbiodinium* spp. from the host coral. Generated through photoreduction of oxygen in either photosystem I (PSI) or II (PSII), ROS are prevented from proliferating throughout the thylakoid membrane and into external tissues by antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) (Asada, 2006; Smith,

Suggett, & Baker, 2005). However, imbalances in the capacity for suppression of photosynthetic pro-oxidant products due to thermal stress and synergistic impacts by other environmental pressures, such as UV radiation, can lead to excessive reactive oxygen production, outstripping the ability for the symbiont to detoxify tissues (Asada, 2006; Gates et al., 1992; Lesser, 1996). Subsequent ROS diffusion, mainly in the form of hydrogen peroxide, which is able to passively diffuse through cellular membranes, can induce oxidative damage to coral host tissues, (Davies, 1987; Lesser, 1996, 1997). As suggested by Lesser (1997), increased antioxidant presence can aid in the reduction of oxidative stress and a return to pre-stress levels of photosynthetic activity in the coral holobiont. Since antioxidant enzymes are a basal component of detoxifying the tissues of the symbiont and coral host, their role in the identification of stress leading to a bleaching response makes them effective tools for characterizing molecular imbalances initiating a physical stress response in corals.

Previous studies have used various components of the pro-oxidant enzyme detoxification network as proxies for studying the metabolic responses different corals exhibit to thermal stress (Desalvo et al., 2008; Flores-Ramírez & Liñán-Cabello, 2007; Lesser, 1997; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010; Yakovleva et al., 2004). In their 2002 study, Downs et al. found concentrations of SOD within the tissues of *Montastraea annularis* colonies increased in accordance to months with highest temperature. Further, in the coral *P. verrucosa*, Rodriguez-Troncoso, Carpizo-Ituarte, & Cupul-Magaña (2013) observed that CuZn SOD activity significantly increased with increasing temperature. However, it must be noted that responses to heat stress can also be species specific, as research conducted by Flores-Ramírez & Liñán-Cabello (2007) illustrated, with *P. capitata* failing to exhibit similar significant increases in SOD activity as seen in *P. verrucosa* with respect to increasing heat. Further, variable expression in these bioindicators of oxidative stress were also shown to occur between different genera (Yakovleva et al., 2004). This suggests that there are multiple levels of regulation within and between different species and genera, to counter thermal stress. This would confer variable tolerance to heat stress by utilizing different antioxidant defense pathways or levels of antioxidants. In addition, there is also a need to explore various routes of antioxidant enzyme response to illustrate a more complete view of the detoxification

process. As such, although studies into the responses of single species to thermal stress may be useful to guide the development of biomarkers for general use, it is also necessary to determine how individual species cope with stress. In this manner we can establish more accurate models for coral susceptibility to changing thermal norms, both globally and locally.

Whilst acknowledging biological hurdles to developing better diagnostic tools, by performing this research we seek to characterize the presence and activity of antioxidant enzymes over a bleaching cycle in the coral *P. damicornis*. *Pocillopora damicornis* is a commonly studied coral, with wide global distribution, and is an appropriate model in which to characterize response to thermal exposure (Griffin et al., 2006; Hoeksema et al., 2014; Lesser, 1996; Rivest & Hofmann, 2014; Sogin, Putnam, Anderson, & Gates, 2016; Veron, 2000). Furthermore, thermally-induced oxidative stress responses in this coral species have not been fully characterized. Hence, we have an outstanding opportunity to improve scientific and environmental knowledge. In addition, this work can validate the utility of antioxidant enzymes such as: CAT, glutathione peroxidase (GPx), glutathione reductase (GR), and SOD as biomarkers of coral health.

The goal of this study is to provide the coral reef conservation community with two major contributions. These being improved insight into the molecular behavior of corals at the peak of heat-induced stress and their recovery, and an improved range of molecular tools to employ towards evaluating stress profiles in corals and identifying recovery on molecular scales.

Identification of the degree of antioxidant defensive enzyme response within *P. damicornis* across a thermal spectrum from pre-exposure to recovery will aid in elucidating how this coral copes with the cycle of stress exposure over a full bleaching period.

Role of Symbiodinium spp. Clade on Coral Health and Implications of Mass-Bleaching

Zooxanthellate hermatypic corals are complex and intricate organisms with widespread distributions that form the backbones of tropical marine ecosystems (Morrissey, Sumich, & Pinkard-Meier, 2018). Their ecological success within marine benthic habitats is closely tied to the photosynthetic efficiencies of their endosymbiotic

dinoflagellates (zooxanthellae)(Levinton, 1995). Providing corals with up to 90% of their metabolic needs, this mutualistic relationship is vital for their survival in the oligotrophic environments they colonize (Birkeland, 1997; Morrissey et al., 2018). In addition to nutrient provision, this symbiosis also offers corals with partial resistances to factors that could otherwise limit survivorship and growth, such as hypoxia (Rands et al., 1992), high and low irradiance (Baker, 2001), and high temperature (Berkelmans & van Oppen, 2006; West & Salm, 2003). However, this symbiosis is sensitive to prolonged environmental anomalies, especially thermal stress (Gates et al., 1992; Glynn & D'Croz, 1990; Hoegh-Guldberg, 1999; Jokiel & Coles, 1977, 1990). This puts corals at a great risk for widespread mortality, as global environmental changes bring forth a wave of what will eventually become chronic stressors on their health (Frieler et al., 2012; Gates et al., 1992; Hughes et al., 2017; Kaniewska et al., 2012; Maynard et al., 2015). Recent work has illustrated the potential for corals to increase their thermal tolerance by associating with certain clades of *Symbiodinium* (Cunning, Silverstein, & Baker, 2015; Silverstein et al., 2015). As global heating incidences increase, these shifts towards thermo-tolerant symbiont recruitment may hold a key for the survival of modern coral reefs.

Corals are capable of reducing cellular stress through upregulation of enzymes and other molecules, which combat damaging chemicals and their metabolites that are produced under thermal stress (Flores-Ramírez & Liñán-Cabello, 2007; Higuchi et al., 2008; Souter et al., 2011). For example, algal symbionts under heat stress produce potentially cytotoxic molecules, such as reactive oxygen species (ROS). Coral stress response to increased ROS helps reduce damages that can lead to bleaching under prolonged heat exposure (Lesser, 1996; Rodriguez-Troncoso et al., 2013). In addition to the coral host's defenses, ROS can also be mitigated by symbionts through their specialized antioxidant enzymes (Richier et al., 2005). However, findings from Barshis, Ladner, Oliver, & Palumbi (2014) suggest that the capacity of *Symbiodinium* spp. to respond to thermal stress may be limited, as no significant transcriptional changes were detected in symbionts under prolonged heat exposure. Furthermore, the lack of transcriptional changes was observed in both the less thermally tolerant clade (clade C) and the thermo-tolerant clade (clade D) (Barshis et al., 2014). Such findings support the

hypothesis that mounting oxidative stress must be primarily mitigated by the coral host, eventually leading to the evacuation of the ROS source, the zooxanthellae (Gates et al., 1992). Although no significant differences in gene expressions were detected between the two clades of *Symbiodinium* under thermal stress in Barshis, Ladner, Oliver, & Palumbi (2014) found significant transcriptional differences of certain orthologous genes between the two clades. Such transcriptional differences existed even without heat exposure (Barshis et al., 2014), which supports the hypothesis that response limits to environmental stress may drive the host-symbiont clade pairings (Cunning, Silverstein, et al., 2015; Silverstein et al., 2015; Stat et al., 2008).

There are nine defined clades of *Symbiodinium* (A-I), and their subclades associated with corals (Pochon & Gates, 2010; Pochon, Montoya-Burgos, Stadelmann, & Pawlowski, 2006). The levels of stress tolerance and photosynthetic efficiency differ among the clades, which influences corals' long-term health, reproduction, and growth (Barshis et al., 2014; Berkelmans & van Oppen, 2006; Cunning et al., 2016; Cunning, Silverstein, et al., 2015; Rands et al., 1992; Rowan, Knowlton, Baker, & Jara, 1997; Stat et al., 2008). For example, *Acropora tenuis* and *A. millepora* experienced up to 3 times faster growth when harboring *Symbiodinium* clade C versus clade D (Little, van Oppen, & Willis, 2004). Similar trends were seen in *P. damicornis*, where colonies harboring clade D grew 35-40% slower than those harboring clade C (Cunning, Gillette, Capo, Galvez, & Baker, 2015). However, when exposed to both extreme heat and cold, corals with clade C exhibited low thermal tolerances, and subsequently had a high level of partial to complete bleaching, while those with clade D either did not bleach, or bleached at a significantly lower rate (Cunning, Silverstein, et al., 2015; Glynn, Maté, Baker, & Calderón, 2001; LaJeunesse et al., 2010; Silverstein et al., 2017). *Symbiodinium* belonging to clade A has been categorized as potentially ‘parasitic’ in Pacific corals due to significantly lower fixed carbon output compared to the colonies hosting clade C (Stat et al., 2008). Clade B has been noted to be opportunistic in colonizing *Pocillopora* spp. directly after bleaching, but quickly replaced by those belonging to other clades after recovery (LaJeunesse et al., 2010). However, not all clades have similar functions across different species and regions. A prominent example is the affinity of Caribbean corals to harbor *Symbiodinium* clades A and B (Baker, 2003; LaJeunesse, 2005). In these

associations, clade A appears to have adopted the role of a thermo- and UV-tolerant symbiont that is commonly associated with clade D in Pacific corals (Baker, 2003; Suggett et al., 2008). Different benefits to coral hosts brought by different symbiont clades highlight the importance of securing optimal symbionts to maximize the fitness of a colony. Flexible symbiont associations could also have a substantial influence on coral health.

Some *Symbiodinium* clades are predominantly associated with certain species of corals, and some coral species show no shuffling of clades even after bleaching (Cunning, Glynn, & Baker, 2013; Glynn et al., 2001; LaJeunesse et al., 2010; McGinley et al., 2012; Stat et al., 2008). The genera *Pocillopora, Pavona, Porites, Gardineroseris,* and *Psammocora* are known to have high fidelity to certain clades of symbionts without shuffling (Glynn et al., 2001; McGinley et al., 2012). For example, in eastern Pacific populations of *Pocillopora* spp., less than 3% of surveyed colonies changed their symbionts from thermally-sensitive clade C to thermally tolerant clade D (McGinley et al., 2012), while some genera would undergo full shifts following prolonged thermal exposure (Silverstein et al., 2015). However, for *Pocillopora* spp, conflicting results also exit; *Pocillopora* colonies that recruited more thermally tolerant symbionts, or in which the dominant clade was thermo-tolerant, were observed to be more abundant and more resilient in the mass bleaching events (Glynn et al. 2001; Baker et al. 2005).

During late 2014, coral reefs throughout the Hawaiian Archipelago underwent a significant bleaching event due to sustained temperatures over 27°C (Bahr et al., 2015; Cunning et al., 2016), which brought an opportunity to further characterize the effect of thermally-induced bleaching on clade shuffling in the coral *P. damicornis. Pocillopora damicornis* has broad distribution throughout Indo-Pacific (Hoeksema et al., 2014), and Pocilloporid corals were most affected by the 2014 bleaching event, with *P. damicornis* experiencing highest mortality over other surveyed corals within the study site, Kāneʻohe, Oʻahu, Hawaiʻi (Bahr et al., 2015). Due to their sensitivity to thermal stress, and in addition to our studies presented in findings from our investigation of reproductive effects on enzyme activity, analyzing the pre- and post-bleaching symbiont clade would provide further insight into adaptive capacity of *P. damicornis* to thermal stress.

Hypotheses

To address the goals of this comprehensive study investigating reproductive baselines for antioxidant enzyme expression, characterization of the response of *P. damicornis* to bleaching stress and recovery post-thermal event, and the effect of thermally-induced bleaching on symbiont clade recruitment, we tested five main null hypotheses:

1) reproductive cycling has no effect on antioxidant enzyme activity
2) reproductive cycling has no effect on antioxidant enzyme expression
3) there will be no bleaching stress effect on enzyme activity
4) there will be no bleaching stress effect on enzyme expression, and
5) there will be no bleaching stress effect on symbiont clade recruitment

Chapter 3: Methodology

Sample Collection

Coral samples (5 cm x 2.5 cm nubbins) were collected periodically from the same 6 colonies off Lilipuna Pier, Kāneʻohe, Oʻahu, Hawaiʻi (figure 4), adjacent to the Hawaiʻi Institute of Marine Biology (HIMB).

Figure 4. Site map denoting locations of the 6 Pocillopora damicornis colonies of interest in this study (Kāneʻohe, Oʻahu, Hawaiʻi).

Reproductive Enzyme Variation Sampling

To reduce the impact of experimental fragmentation on the reproductive cycling or output of *P. damicornis* (Zakai, Levy, & Chadwick-Furman, 2000), colonies were not fragmented prior to the start of collections. Instead, fragments of branches were sampled from 2 areas (distal to each other) on each colony to ensure reduction of microhabitat influence and intracolony stress load variation between samples. This was also done to limit variations in reproduction potential along coral branches (polyps found mid-branch retain the highest planula larvae output versus distal and central branch polyps) and sampling was also conducted during falling tides to both reduce residence time in low-flow water and match peak planula release, as it has been correlated with low tide periods

(Harrison & Wallace, 1990). Colonies with minimal competition from other corals and no visible signs of disease or stress were chosen for sampling, and samples were taken during new, ¼, full, and ¾ moon phases during July and August 2014 (n = 6 per sampling period). Collections also included an acute sampling period, during which corals were sampled daily for five consecutive days following the start of the peak reproductive period moon phase (¼ moon) in the month of August (n = 6 per sampling period). This sampling period was integrated into the experimental design to provide finer resolution for understanding changes in antioxidant enzyme profiles following a reproductive peak.

Sampling was designed to illustrate variations in enzymatic activity within the moon phase cycle and fragments were immediately frozen in liquid nitrogen to preserve enzyme profiles and protein integrity. Samples were transported to Kewalo Marine Laboratory (KML) on dry ice and transferred to a -80°C freezer. Upon returning to KML, corals were crushed into a fine powder on liquid nitrogen and returned for storage in a VWR 5656 -80ºC freezer (Radnor, PA, USA) until further processing.

Bleaching Cycle Sampling

For the purpose of studying bleaching cycle effects on coral health, colonies were sampled when visually bleached (little to no tissue pigmentation) during the 2014 heating anomaly (Bahr et al., 2015) and four months later to measure post-bleaching stress profiles and monitor potential symbiont variation. Samples were collected during the ¼ moon cycle in October 2014 (bleaching) and February 2015 (post-bleaching), and consisted of sampling from the colonies "1, 2, 5, and 6" (n = 4) employed in the investigation of antioxidant enzyme expression in accordance with reproductive cycling. Colonies 3 and 4 were excluded from this analysis, as these colonies died following the onset of bleaching. Samples for bleaching (n = 4) and post-bleaching (n = 4) were compared to those collected during the ¼ moon cycle in July 2014 (n = 4), three-months prior to peak bleaching in sampled colonies. Sampling was consistent with sampling for the examination of reproductive effects on antioxidant enzyme expression, where fragments were collected from 2 locations (distal to each other) on each colony to reduce microhabitat influence on expression profiles. This was also conducted to limit intra-

colony variation on bleaching stress expression and the distribution of symbiont clade in each specimen (Rowan et al., 1997). Routine sampling was accomplished by removing fragments from colonies during similar tidal periods and before peak irradiance to limit environmental influences on colony health as much as possible. Additionally, sampled corals were immediately frozen in liquid nitrogen to preserve sample integrity before transporting samples to KML for storage at -80°C.

Traditional Cultural Knowledge Sampling Protocol

Due to the location of this research and cultural ties of this researcher, Hawaiian ‘oli, or chants, were integrated into the collection protocol. Without a written language, Hawaiians employed ‘oli as a means of passing down knowledge in the form of orally communicated genealogies, stories, and protocols for interacting with specific daily or ceremonial practices, among other things. Prior to each collection, “E Hō Mai” and “Nā ‘Aumākua” were chanted to ask for knowledge and permission to enter the collection site, while “‘Oli Mahalo” was chanted following each collection to both signify the end of the sampling period and give thanks for the coral taken (see appendix).

S9 Protein Fraction Extraction/Zooxanthellae Isolation and Protein Quantification

Prior to protein isolation, coral samples were crushed into a fine powder using liquid nitrogen and an arbor press. Following modified protocols by Lesser et al. (1990), coral S9 post-mitochondrial protein fractions and zooxanthellae were isolated from crushed coral tissue. Using 1500 mg of crushed tissue and 1500 µL of homogenization buffer per sample extraction in 50 mL tubes (0.01 M Tris-HCl buffer pH 8.0, 1 M phenylmethylsulfonyl fluoride in 1% v/v dimethyl sulfoxide), tissue was homogenized for 1 minute on ice using an Ultra-Turrax homogenizer. The homogenate was then spun for 5 minutes at 4ºC at 2000 rcf in an Eppendorf Microcentrifuge 5415D (Hauppauge, NY, USA) to separate skeleton and tissue, and the supernatant was transferred to 1.5 mL microcentrifuge tubes and spun for 20 minutes at 4ºC at 10,000 rcf. The supernatant was then aliquoted into 1.5 mL tubes and zooxanthellae pellets were saved, and frozen at -80ºC; 50 µL of each extracted sample was set aside for protein concentration analyses.

In preparation for enzymatic activity assays, protein concentrations from each sample were measured using a bicinchoninic acid (BCA) assay. The standard curve was constructed with bovine serum albumin from 0 to 1.0 mg/mL protein (25 µL/well in triplicate), 1:5 dilutions of aliquots from each extracted S9 sample fraction in double distilled water (ddH_2O) were loaded into a 96 well plate in triplicate (25 µL/well). Bicinchoninic acid development reagent (2% $Cu^{2+}SO_4$ in BCA; Sigma-Aldrich) was then added into each well (200 µL/well), and the loaded plate was incubated at 37ºC for 30 minutes. Following incubation, absorbance values were determined at $\lambda = 562$ nm in a SpectraMax M5 Micro-Plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). To ensure triplicate absorbance variation was within acceptable experimental levels (percent coefficient of variation, %CV < 10%; coefficient of determination, $R^2 > 0.98$), data were then exported to Microsoft Excel and %CV and subsequent standard curve R^2 values were calculated. Sample protein concentration values were then interpolated from the standard curve; those extractions falling below 1 mg/mL required re-extraction of S9 post-mitochondrial protein fractions.

Enzyme Activity Assays

Enzyme assays were developed in-house for CAT, GR, SOD, and GPx, and chemicals for assays were sourced from Sigma-Aldrich (St Louis, MO, USA), BioVision (Zurich, Switzerland), EMD Millipore (Burilington, MA, USA), and Cayman Chemical (Ann Arbor, MI, USA). Assays were analyzed using a SpectraMax M5 Multi-Plate reader and final activity values calculated using SoftMax Pro and Microsoft Excel.

The metabolism of H_2O_2 as a marker for CAT activity was accomplished by measuring the consumption of H_2O_2 over time by analyzing decreasing absorbance of H_2O_2 at $\lambda = 240$ nm. Briefly, the method was performed as follows: on ice, coral protein extractions were first diluted to 1 mg/mL in 50 mM potassium phosphate buffer pH 7.0 and then loaded, in triplicate (10 µL/well), into optically clear microtiter 96-well plates; negative controls were also run in triplicate containing all assay reagents except S9 protein to correct for spontaneous H_2O_2 degradation during activity reads (table 1). Running buffer (50 mM potassium phosphate buffer pH 7.0) was then loaded into wells (80 µL/well), and samples were incubated for 3 minutes at 25ºC. The CAT activity

reaction was then initiated by adding 10 µL of 120 mM H_2O_2 to each well, and immediately transferring the reaction plate into the spectrophotometer to read at 10 second intervals for 5 minutes. In order to dislodge O_2 bubbles created by the dismutation of H_2O_2 into H_2O and O_2, the spectrophotometer was set to vibrate the 96-well plate between 10 second reads for 2 seconds; this aided in preventing O_2 bubbles from obscuring the plate reader's evaluation of H_2O_2 absorbance in the reaction wells.

Table 1. Catalase enzyme kinetic assay; 1 mg/mL coral protein, 0.05 M potassium phosphate (KPi) buffer pH 7.0, 120 mM H_2O_2. Assays run in triplicate and wells loaded in sequential order from top to bottom; reference and coral blank triplicates run to account for spontaneous degradation and endogenous levels of H_2O_2 in wells and samples, respectively.

Reference	Coral Sample	Coral Blank
-	10 µL Sample	10 µL Sample
90 µL KPi Buffer	80 µL KPi Buffer	90 µL KPi Buffer
Incubate at 28°C for 3 minutes		
10 µL H_2O_2	10 µL H_2O_2	-

To evaluate the activity of GR, the consumption of NADPH at $\lambda = 340$ nm was observed over time as GR in coral samples consumed this co-factor during the reduction of the reagent oxidized glutathione (GSSG). In order to account for both spontaneous degradation of NADPH in reaction wells and endogenous concentrations of NADPH in coral samples, wells containing no coral sample (spontaneous degradation control), and those with coral sample but no NADPH (background level control), were also evaluated alongside wells containing all reagents; in place of coral sample and NADPH, an extra 20 µL of 100 mM potassium phosphate buffer (pH 7.2) was added to wells in the first assay step (table 2). Values for NADPH degradation obtained from these controls were subtracted from overall activity following assay completion. In optically clear microtiter 96-well plates, 100 mM potassium phosphate buffer was loaded into wells (130 µL/well, in triplicate), followed by 100 mM ethylenediaminetetraacetic acid (EDTA) in ddH_2O (10 µL/well), 10 mM GSSG in ddH_2O (20 µL/well), 1.2 mM NADPH in ddH_2O (20 µL/well), and 1 mg/mL coral sample (20 µL/well). Plates were then loaded into the spectrophotometer and mixed using its mixing function for 5 seconds. Absorbance reads were conducted at 20 second intervals for 5 minutes at 25ºC.

Table 2. Glutathione reductase enzyme kinetic assay protocol; 1 mg/mL coral protein, 100 mM KPi buffer pH 7.2, 100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM oxidized glutathione (GSSG), 1 mg/mL nicotinamide adenine dinucleotide phosphate (NADPH). Assays wells loaded in triplicate with accordance to order of reagents from top to bottom; reference and coral blank triplicates run to account for spontaneous degradation and consumption of endogenous levels of NADPH in assay mixture and coral samples, respectively.

Reference	Coral Sample	Coral Blank
150 µL KPi Buffer	130 µL Working Buffer	150 µL Working Buffer
10 µL EDTA Stock Sol'n	10 µL EDTA Stock Sol'n	10 µL EDTA Stock Sol'n
20 µL GSSG Sol'n	20 µL GSSG Sol'n	20 µL GSSG Sol'n
20 µL NADPH Sol'n	20 µL NADPH Sol'n	-
-	20 µL Sample	20 µL Sample

Since SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2, this assay conducted an indirect evaluation of SOD activity by analyzing the degree of inhibition of the reduction of cytochrome c by O_2^-. Using the following loading protocol (table 3), reagents and samples were loaded into a 96-well microtiter plate in triplicate, loaded into and mixed using the mixing function in the spectrophotometer for 5 seconds, and analyzed for decreasing absorbance (λ = 550 nm) for 5 minutes in 20 second intervals at 25ºC. For this assay: working buffer consisted of 100 mM potassium phosphate buffer (pH 7.8), 0.2 mM EDTA in ddH_2O, 100 µM hypoxanthine in ddH_2O, and 20 µM cytochrome c in ddH_2O; samples were diluted to 1 mg/mL in 100 mM potassium phosphate buffer (pH 7.8); and reactions were initiated with the addition of 300 mU/mL of O_2^- generating xanthine oxidase in ddH_2O.

Table 3. Superoxide dismutase enzyme kinetic assay protocol; 1 mg/mL coral protein, 100 mM KPi buffer 7.8, 300 mU xanthine oxidase, working buffer: 100 mM KPi buffer, 0.2 mM EDTA, 100 µM hypoxanthine, 20 µM cytochrome c. Assay well reagents loaded in triplicate sequentially from top to bottom. Reference and coral blank triplicates run to account for spontaneous degradation and endogenous levels of cytochrome c in assay mix and in coral samples, respectively.

Reagent	Reference	Coral Sample	Coral Blank
KPi Buffer	80 µL	74 µL	94 µL
Working Buffer	100 µL	100 µL	100 µL
Coral Sample	-	6 µL	6 µL
Initiate Reaction			
Xanthine Oxidase	20 µL	20 µL	-

To evaluate the activity of GPx, assays were broken into two parts in order to determine the activity of both selenium-dependent and selenium-independent forms of

this enzyme. As such, table 4 illustrates the loading protocol in place for evaluating the activity of selenium-dependent GPx, using H_2O_2 as the initiator and substrate for this reaction, including sodium azide (NaN_3) to inhibit catalase activity from consuming H_2O_2 and interfering with assay results. To evaluate the activity of selenium-independent GPx, cumene hydroperoxide (CHP) was utilized in place of H_2O_2 (table 5). In order to achieve the breakdown of their substrates, GPx uses reduced glutathione (GSH) as the cofactor for hydroperoxide reduction, producing oxidized glutathione as the final product (GSSG). In order to visualize this breakdown and quantify GPx activity, this assay has been adapted to measure the consumption of reduced NADPH by GR to replenish GSH from the GPx by-product, GSSG. By this method, measured decreases in NADPH is proportional to GPx activity, which is monitored at $\lambda = 340$ nm for 5 minutes at 20 second intervals in optically clear microtiter 96-well plates. Assay reagents consisted of: 100 mM potassium phosphate buffer (pH 7.0), 20 mM NaN_3 working solution in ddH_2O, 100 mM EDTA in ddH_2O, 100 mM GSH working solution in ddH_2O, 100 U/mL GR working solution in ddH_2O, 1 mg/mL coral samples in 100 mM potassium phosphate buffer (pH 7.0), 1.25 mg/mL NADPH working solution in ddH_2O, 150 mM H_2O_2 working solution, and 150 mM CHP working solution. Three sets of reference wells were run to account for degradation of assay substrates over time (no coral sample with substrates), non-specific oxidation of NADPH in this assay (no H_2O_2 or CHP), and endogenous levels of substrate in coral tissue samples (coral sample with no substrate). Upon loading plates into the spectrophotometer, plates were mixed, and absorbance changes immediately read.

Table 4. Se-dependent GPx enzyme kinetic assay protocol; 1 mg/mL coral protein, 100 mM KPi buffer pH 7.0, 20 mM sodium azide, 100 mM EDTA, 100 mM reduced glutathione, 100 U/mL glutathione reductase, 1.25 mg/mL NADPH, 150 mM H_2O_2. Assay wells loaded in triplicate with reagents sequentially, from top to bottom. Coral blank and H_2O_2 blanks used to account for endogenous levels and non-specific oxidation of NADPH in this assay.

Reagent	**Reference**	**No H_2O_2**	**Coral Sample**	**Coral Blank**
KPi Buffer	130 µL	140 µL	110 µL	120 µL
NaN_3 Working Sol'n	10 µL	10 µL	10 µL	10 µL
EDTA	10 µL	10 µL	10 µL	10 µL
GSH Working Sol'n	20 µL	20 µL	20 µL	20 µL
GR Working Sol'n	10 µL	10 µL	10 µL	10 µL
Coral Sample	-	-	20 µL	20 µL
Instrument Autozero				
NADPH Working Sol'n	10 µL	10 µL	10 µL	10 µL
Initiate Reaction				
H_2O_2 Working Sol'n	10 µL	-	10 µL	-

* H_2O_2 blank is used to account for non-specific oxidation of NADPH in this assay

Table 5. Se-independent GPx enzyme kinetic assay protocol; 1 mg/mL coral protein, 100 mM KPi buffer pH 7.0, 100 mM EDTA, 100 mM reduced glutathione, 100 U/mL glutathione reductase, 1.25 mg/mL NADPH, 150 mM cumene hydroperoxide (CHP). Assay well loaded in triplicate with reagents sequentially, from top to bottom. Coral blank and CHP blanks used to account for endogenous levels and non-specific oxidation of NADPH in this assay.

Reagent	**Reference**	**No CHP**	**Coral Sample**	**Coral Blank**
KPi Buffer	140 µL	150 µL	120 µL	120 µL
EDTA	10 µL	10 µL	10 µL	10 µL
GSH Working Sol'n	20 µL	20 µL	20 µL	20 µL
GR Working Sol'n	10 µL	10 µL	10 µL	10 µL
Coral Sample	-	-	20 µL	20 µL
Instrument Autozero				
NADPH Working Sol'n	10 µL	10 µL	10 µL	10 µL
Initiate Reaction				
CHP Working Sol'n	10 µL	-	10 µL	-

* CHP blank is used to account for non-specific oxidation of NADPH in this assay

Western Immunoblotting

Materials for running Western Immunoblotting protocols were sourced in-house and through Li-Cor, and Western Blots were read using a Li-Cor C-DiGit Blot Scanner (Lincoln, NE, USA). Antibodies for SOD-1, CAT, GPx, and GR were sourced from Santa Cruz Biotechnology (Dallas, TX, USA) and preliminary testing with *P. damicornis* found all antibodies to work in this coral species.

Preliminary range-finding experiments found that coral protein concentrations of 60 ng provided adequate resolution for visualizing protein bands for all target proteins.

As such, samples were appropriately diluted to 60 ng with Laemmli loading buffer (1.5 M Tris-HCl pH 6.8, 50% v/v glycerol, 25% v/v β-mercaptoethanol, 0.35 M SDS, 1% v/v 1% bromophenol blue), boiled for 5 minutes at 95ºC, spun for 1 minute at top centrifuge speed, and then loaded into 10% polyacrylamide gels along with PageRuler Plus protein ladder (ThermoFisher Scientific); gels were then run for 1.5 hours at 80 volts (V). To note, all samples were run in parallel with companion gels to ensure technical replicates to ensure results were not caused by technical errors in the blotting analysis process.

After electrophoresis, gels were removed from running tanks and transferred into transfer buffer (25 mM Tris Base, 0.192 M glycine, 20% v/v 100% methanol, in ddH_2O) to remove Tris-Glycine SDS-PAGE running buffer (25mM Tris Base, 0.192M glycine, 3.5 mM SDS, in ddH_2O). Blotting cassettes were then prepared for transfer, soaking blotting paper and sponges in chilled transfer buffer, while 0.2 um polyvinylidene difluoride (PVDF) membranes were activated in 100% methanol. Taking soaked blotting sponges and paper out of transfer buffer, transfer cassettes were set up in the following order: blotting sponge, blotting paper, gel, PVDF membrane, blotting paper, blotting sponge. Cassettes were then placed in transfer tanks, tanks were filled with transfer buffer and a frozen ice pack, moved into the 4ºC refrigerator, and transferred overnight at 40V.

To confirm complete protein transfer and evaluate for equal loading, gels and membranes were then stained with Coomassie blue R-250 and Ponceau S, respectively, before primary antibody (1º ab) incubation. With respect to checking for complete transfer of target proteins, gels were incubated for 1 hour in fixing solution (50% v/v 100% methanol, 10% v/v glacial acetic acid, in ddH_2O), then stained for 2 hours in staining solution (0.25% w/v Coomassie brilliant blue R-250, 50% v/v 100% methanol, 10% v/v glacial acetic acid, in ddH_2O), and then immersed in de-staining solution (5% v/v 100% methanol and 7.5% v/v glacial acetic acid, in ddH_2O) for 4 hours to reduce background dying. Stained gels were then visualized to assess proteins transfer.

Alternatively, membranes were first rinsed in ddH_2O three times, and then rinsed in 1X Phosphate Buffered Saline with Tween 20 (PBST, pH 7.4)(0.14 M sodium chloride, 1.34 mM potassium chloride, 10.14 mM disodium phosphate, 1.76 mM monopotassium phosphate, 0.05% v/v Tween-20, in ddH_2O) for 5 minutes. Membranes were then immersed in Ponceau S solution (BioReagent) for 5 minutes. Following

membrane staining, membranes were then de-stained in gel storage solution (5% v/v glacial acetic acid, in ddH_2O) for two 5 minute washes, changing solution each time. Membranes were then scanned for protein transfer and equal loading visualization. Finally, membranes were transferred into two 5 minute washes in PBST, changing the PBST each time, and then blocked in 5% milk (5% w/v powdered milk, in ddH_2O) for 1 hour.

Following the blocking process, membranes were washed for 15, 10, 5, and 5 minutes in PBST, changing the PBST each time. Primary antibodies (1º ab) were then prepared in 1:1000 dilutions, and membranes were incubated protein side up with their respective 1º ab overnight at 4ºC on a rotary table. The next day, membranes were washed in PBST for 15, 10, 5, and 5 minutes, changing PBST each time. Secondary antibodies (2º ab) were then added (1:2000 dilution) to membrane blotting boxes, and membranes were incubated on a rotary table for 2 hours at room temperature. After 2º ab incubation, membranes were again rinsed for 15, 10, 5, and 5 minutes in PBST, changing PBST with each wash, and then prepared for visualization.

A Li-Cor C-Digit Blot Scanner was utilized to read membranes. For scanning, membranes were dried face-up for 1 minute, during which membrane labels and ladder bands were marked with Li-Cor WesternSure Pen ink for scan imaging. Membranes were then placed face-down onto 1:1 WesternSure ECL Substrate for 1.5 minutes (1 part Luminol Enhancer Solution and 1 part Stable Peroxidase Solution; Li-Cor). Using forceps, membranes were then placed protein side down on the blot scanner and read for 12 minutes. Choosing the image with best resolution for the targeted protein, images were then adjusted for brightness and background reduction, and membranes returned to PBST and either stored at 4ºC or prepared for antibody stripping. Between scans, the blot scanner was wiped down with Kimwipes to prepare for the next membrane.

If being stripped for the next 1º ab, membranes were placed in strong stripping buffer (100 mM β-mercaptoethanol, 2% w/v SDS, 62.5 mM Tris-HCl pH 6.8, in ddH_2O) in an incubator set to 55ºC for 5 to 10 minutes; time varied based on band intensity during scanning. After stripping, membranes were then rinsed with 3 times with ddH_2O, then washed twice for 5 minutes in PBST, changing the PBST each time. Membranes were then blocked in 5% milk for 20 minutes, followed by an additional 3 ddH_2O washes

and subsequent 15, 10, 5, and 5 minute washes in PBST, changing the PBST with each wash. Membranes were then incubated overnight following the aforementioned 1º ab protocol.

Due to the use of samples that may have little to no enzyme activity or presence during specific collection time points, various experimental materials and procedural modifications were selected and made to ensure optimal protein band resolution. Due to protein size ranges of 23 to 92 kDa, 10% polyacrylamide gels were chosen to ensure greater separation of smaller protein bands. Gels were also run at a lower voltage for a longer period of time to reduce band smearing and increase resolution. Due to the higher protein binding capacity and durability for multiple stripping and re-probing cycles of PVDF versus nitrocellulose membranes, this material was chosen for this protocol. Further, the 0.2 um membrane pore size was chosen for PVDF membranes versus 0.45 um membranes, as the former's pore size allows for greater sensitivity when detecting proteins of lower molecular weights and proteins that may be loaded at lower levels in running gels. Lastly, overnight transfers and 1º ab incubations were conducted to ensure complete transfers of target protein bands, improve resolution of protein bands on blotting membranes, and improve resolution of bands during the scanning process.

Symbiont Isolation

In accordance with the methodology for preparing samples for protein extraction, symbionts were isolated from coral tissue crushed and powdered on liquid nitrogen. Due to potential variation in symbiont clade due to intra-coral localization (Rowan et al., 1997), powdered samples were shaken following crushing to ensure homogenization of crushed tissues. Zooxanthellae pellets saved from the S9 post-mitochondrial protein fraction extraction process were used to extract DNA using Qiagen DNeasy Blood and Tissue kits (Hilden, Germany).

DNA Isolation, Amplification, and Symbiont Variation Comparison

Running in duplicate, in a 1.5 mL tube, zooxanthellae pellets were mixed with 180 µL of tissue lysis buffer and 20 µL of proteinase K. Samples were vortexed for 15 seconds and incubated overnight at 56°C to ensure complete cell lysis. Following

incubation, samples were vortexed for 15 seconds, and 200 µL of Buffer AL was added to each tube before vortexing again. After vortexing, 200 µL of 100% ethanol was added to each sample, followed by an additional vortexing step. Mixtures were then transferred from their 1.5 mL tubes to DNeasy Mini spin columns mounted in 2 mL collection tubes and tubes were centrifuged for 1 minute at 6000 g; flow-through from this spin and collection tubes were discarded. DNeasy Mini spin columns were then transferred to a new 2 mL collection tube, 500 µL of Buffer AW1 was added, and samples were centrifuged again for 1 minute at 6000 g. Discarding flow-through and collection tubes, 500 uL of Buffer AW2 was added, and tubes were centrifuged to dry DNeasy membranes for 3 min at 20000 g; flow-through and collection tubes were then discarded. DNeasy Mini spin columns were then transferred to new 2 mL tubes, followed by pipetting 20 µL of Buffer AE onto DNeasy membranes. These membranes were then incubated for 1 minute at room temperature, followed by an additional centrifuge step for 1 minute at 6000 g to elute DNA, discarding flow-through and collection tubes following spins. This elution step was repeated again to maximize DNA yield and was followed by quantifying DNA samples using a ThermoFisher Scientific NanoDrop 2000 (Waltham, MA, USA).

Sample DNA was then diluted to 1:20 and run in a 50 µL PCR reaction using symbiont-specific ss3z (5'-AGCACTGCGTCACTCCGAATAATTCACCGG-3') and ss5z primers (equimolar 5'-GCAGTTATAATTTATTTGATGGTCACTGCTAC-3' and 5'-GCAGTTATAGTTTATTTGATGGTTGCTGCTAC-3') to amplify nuclear small subunit (SSU) DNA (Rowan & Powers, 1991; Stat et al., 2008). Polymerase chain reaction, reaction mixture per sample consisted of: 25.6 µL of nuclease-free water, 10 µL 5x Taq reaction buffer, 4 µL $MgCl_2$, 4 µL 2.5 mM dNTPs, 2.5 µL primer ss3z, 2.5 µL primer ss5z, 0.4 µL goTaq, and 1 µL diluted sample DNA. Initial denaturation of samples was conducted for 2 minutes at 95°C (lid temperature 105°C) and amplification of DNA was conducted over 30 cycles (1 minute at 94°C denaturation, 2 minutes at 55°C annealing, 3 minutes at 72°C extension), followed by a final extension for 5 minutes at 72.5°C, holding products at 4°C. To check for amplification of the targeted PCR products, a 2% agarose check gel was run at 70 V for 35 minutes.

Following confirmation that the targeted SSU DNA PCR products were correctly amplified, samples were purified utilizing a MoBio UltraClean PCR Clean-Up Kit

(Carlsbad, CA, USA). To each sample, 225 µL of SpinBind was added and mixed well by pipetting. Samples were then transferred to spin filters inserted on 2 mL microcentrifuge tubes and centrifuged for 30 seconds at 10000 g; guanidine-containing flow-through was collected for waste disposal. Replacing spin filters on associated collection tubes, 300 µL of SpinClean buffer was added to each sample before centrifuging again for 30 seconds at 10000 g; flow-through was again collected for hazardous waste disposal. Samples were then spun for 60 seconds at 10000 g before transferring filter columns to clean 2 mL collection tubes and adding 50 uL of elution buffer (10 mM Tris) solution directly on to the center of the spin filter membranes. Samples were then incubated for 30 minutes before centrifuging a final time for 60 seconds at 10000 g. Final flow-through contained purified restriction fragment length polymorphisms (RFLPs). The purified PCR product concentrations were determined by NanoDrop.

Samples were then digested using *Taq*1 (New England Biolabs, Ipswich, Massachusetts) using the following digest mix for 2 hours at 65°C: 5 µL restriction enzyme buffer, 1 mg DNA, 1 µL *Taq*1 enzyme, and 43 µL nuclease-free water. An 80°C incubation for 20 minutes was then used to inactivate *Taq*1 before holding products at 4°C. Potential RFLP variations were evaluated by diluting samples to 1 µg of DNA and adjusting volumes to 30 uL with nuclease-free water before adding GelGreen dye (Biotium, Fremont, California) and running duplicate samples on partner 2% agarose gels for 40 minutes at 70 V; 1 kB Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA). An additional negative control was run alongside samples to ensure products were not lost during PCR purification or compromised by *Taq*1 digestion.

Statistical Analyses

Enzyme activity value statistical data analyses were conducted using Prism 7.03 (GraphPad Software, La Jolla, CA, USA). Data were entered into "grouped" tables with replicate values in side-by-side subcolumns. Normality of enzyme assay activity values (expressed in "x"mols/min/mg protein) were then evaluated using the D'Agostino and Pearson Omnibus normality test (Supplements). This test first computes the skewness and kurtosis for assay values to assess how entered values differ from Gaussian distribution,

specifically with respect to asymmetry and shape. It then evaluates goodness-of-fit for all enzyme activity values and whether sample data differs from the expected Gaussian distribution. Data were then run through one-way analyses of variance (ANOVA) with Tukey's post-hoc test due to normal Gaussian distribution of the data to elucidate significant differences in enzyme activity values for CAT, GR, GPx, and SOD between reproductive moon phase periods (n = 6), between acute sampling periods (August ¼ moon and following 5 days)(n = 6), and bleaching cycle sampling periods (n = 4); $p < 0.05$, $\alpha = 0.05$, CI = 95%. To accomplish these tests, replicate values were entered into a "grouped" tables with replicate values in side-by-side subcolumns. Significant differences in enzyme activity values were again evaluated between reproductive sampling period (n = 6), between acute sampling periods (n = 6), and bleaching cycle sample periods (n = 4).

Chapter 4: Results

Antioxidant Enzyme Cycling over Reproductive Lunar Cycles in Pocillopora damicornis

Enzymatic Activity Assays

Catalase enzyme kinetic assay analyses displayed a notable trend on enzyme activity cycling over moon phase cycles (figure 5). Although significant variations in enzyme activity were only observed between July Full moon and August ¼ moon sampling periods ($p = 0.0177$, CI = 95%), CAT activity appears to follow a general sinusoidal trend, with activity peaking during the new and ¼ moon phases. No significant variations in enzyme activity were observed during the acute sampling timeline ($p = 0.2374$, CI = 95%; figure 6). This was expected, however, as there was no significant difference between August ¼ and full moon collections.

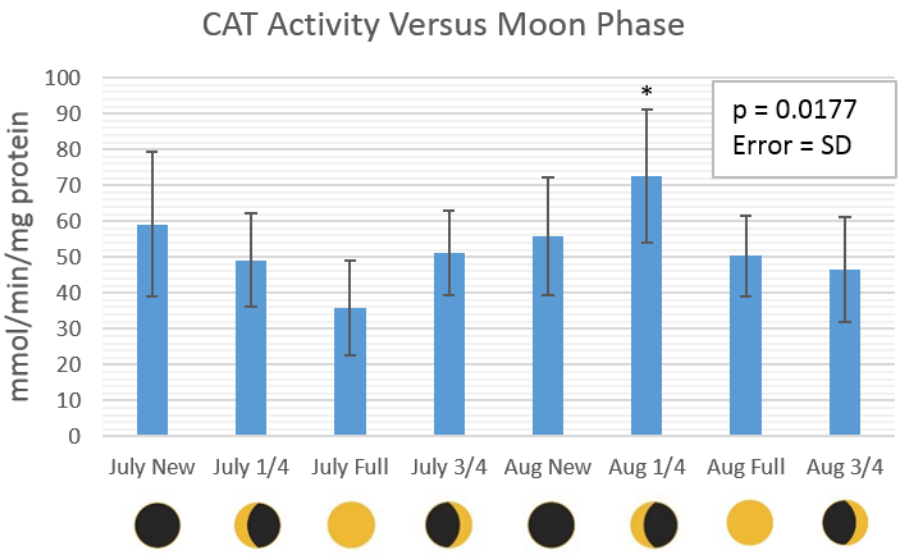


Figure 5. Catalase activity in mmol of H_2O_2 metabolized/min/mg protein versus bleaching cycle; bars represent mean ± SD. Samples collected during Aug ¼ moon expressed significantly higher activity than those collected during the July full moon phase (p = 0.0177, CI = 95%).

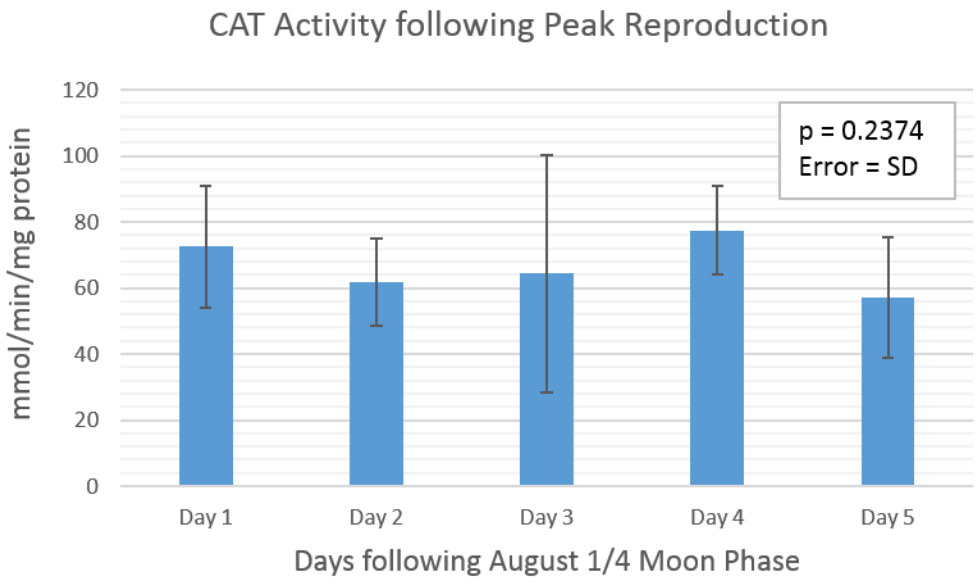


Figure 6. Acute tracking of CAT activity characterized by the consumption of H_2O_2 mmol/min/mg protein versus time following peak reproduction (¼ moon phase); bars represent mean ± SD (p = 0.2374, CI = 95%).

Glutathione reductase activity reflected similar trends as CAT assays, where new and ¼ moon phases harbored higher enzyme activity than comparative full and ¾ moon cycles (figure 7). Activity of GR was significantly higher during July new and ¼ moon and August ¼ moon than those values from full and ¾ moon collection periods (p <

0.0001, CI = 95%). Further, GR activity was found to significantly decrease from day 1 to day 5 of acute sampling following the August ¼ moon (p = 0.0189, CI = 95%, figure 8).

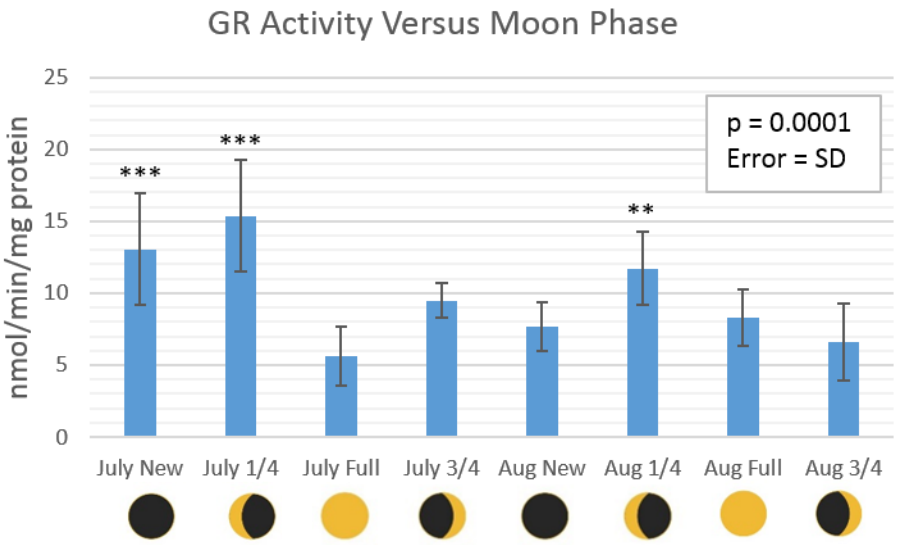


Figure 7. Glutathione reductase activity in nmol of NADPH metabolized/min/mg protein versus bleaching cycle; bars represent mean ± SD (p = 0.0001, CI = 95%). Samples collected during the July New, ¼ and Aug ¼ moon phases displayed significantly higher enzyme activity versus those collected during non-reproductive sampling time points (p < 0.0001).

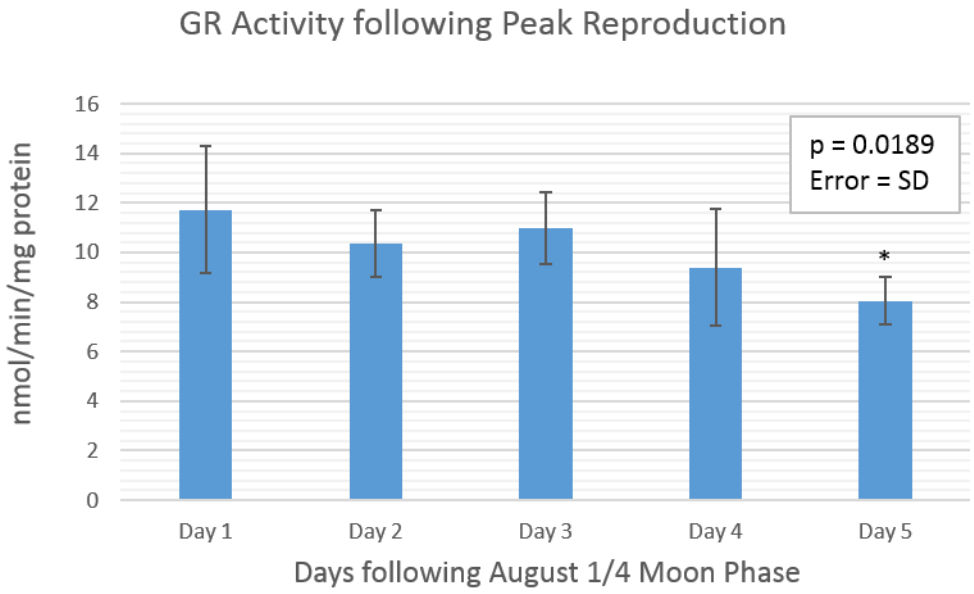


Figure 8. Acute tracking of GR activity characterized by the consumption of NADPH nmol/min/mg protein versus time following peak reproduction (¼ moon phase); bars represent mean ± SD (p = 0.0189, CI = 95%). Activity was found to significantly decrease over the 5 day acute survey period, as activity in samples collected on day 5 were significantly lower than those collected during day 1 of the ¼ moon phase (p = 0.031).

Se-independent GPx activity was found to have significant peaks in activity during both July and August ¾, and August full moon phases ($p = 0.0001$, CI = 95%, figure 9). Inverse to the trends of CAT and GR activity with relation to moon phase cycle, GPx was found to follow a sinusoidal activity curve, with peak activity occurring opposite to *P. damicornis* peak reproductive output. When analyzing values for acute variations in GPx activity following the August ¼ moon, activity significantly increased in the days following the ¼ moon phase (figure 10). However, only activity of August ¼ moon day 3 samples were found to be significantly different than other collection time points, having significantly higher activity than those collected on day 1 of the August ¼ moon ($p = 0.031$, CI = 95%). Interestingly, Se-dependent GPx activity was negligible or not detectable in corals from moon phase and acute collections ($p > 0.05$, CI = 95%, figures 11 and 12, respectively).

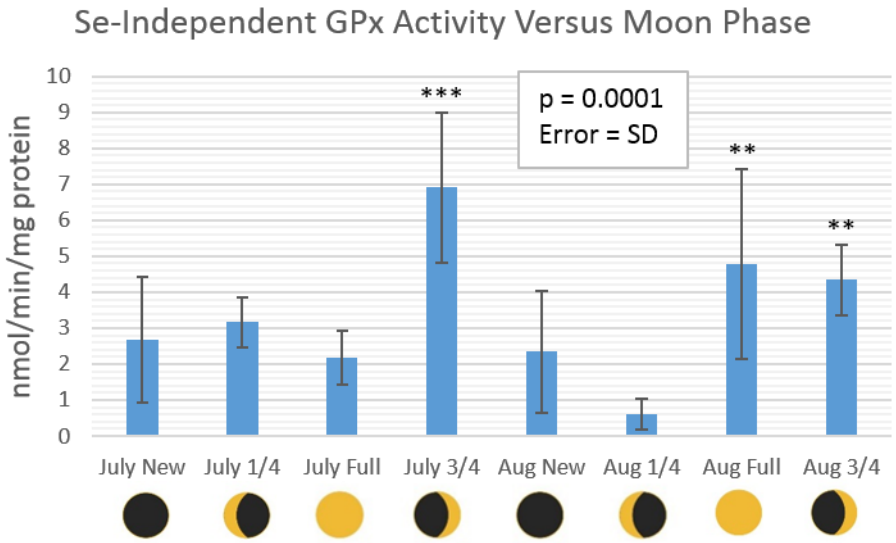


Figure 9. Se-independent GPx activity characterized by the consumption of CHP nmol/min/mg protein versus moon phase cycle; bars represent mean ± SD ($p = 0.0001$, CI=95%). Samples collected during the July ¾ and Aug full, ¾ moon phases displayed significantly higher enzyme activity versus those collected during reproductive sampling time points ($p < 0.0001$).

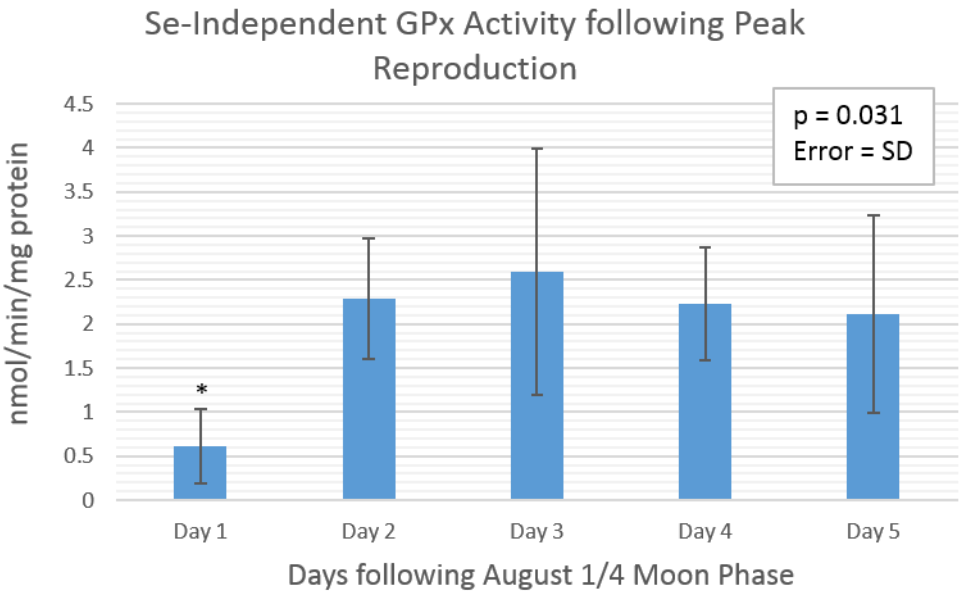


Figure 10. Acute tracking of Se-independent GPx activity characterized by the consumption of CHP nmol/min/mg protein versus time following peak reproduction (¼ moon phase); bars represent mean ± SD (p = 0.031, CI = 95%). Samples collected during day 1 of the ¼ moon phase were found significantly lower in activity versus those samples on day 3 (p = 0.031).

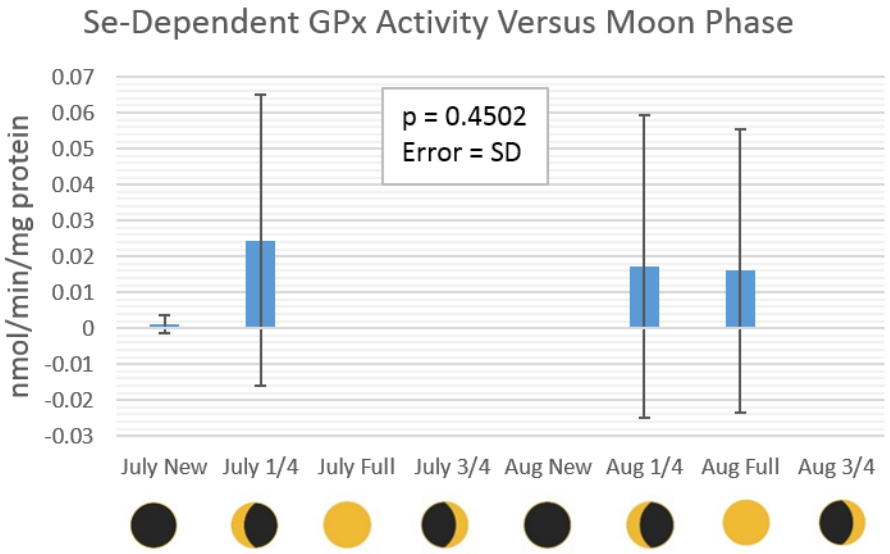


Figure 11. Se-dependent GPx activity characterized by the consumption of H_2O_2 nmol/min/mg protein versus moon phase cycle; bars represent mean ± SD (p = 0.4502, CI=95%).

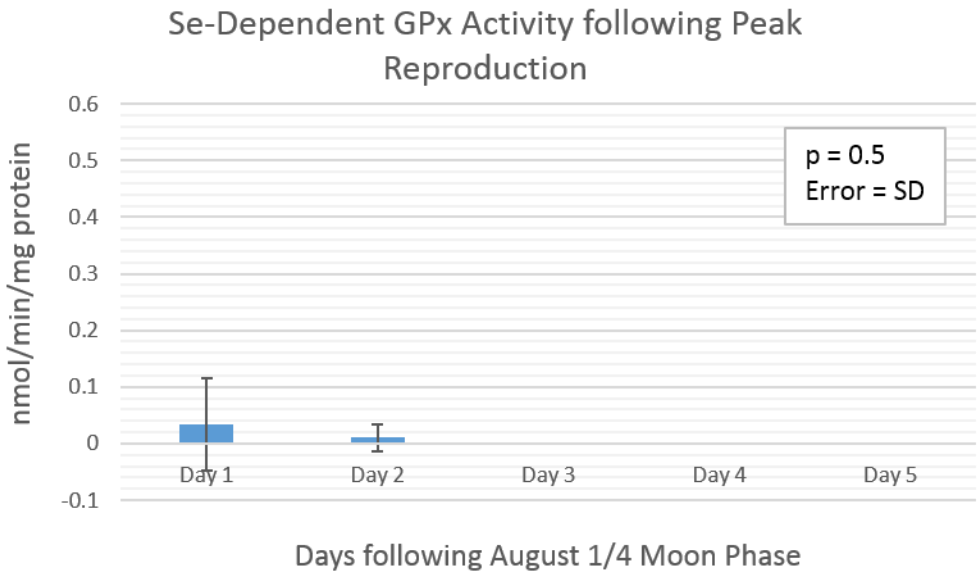


Figure 12. Acute tracking of Se-dependent GPx activity characterized by the consumption of H_2O_2 nmol/min/mg protein versus time following peak reproduction (¼ moon phase); bars represent mean ± SD (p = 0.5, CI = 95%).

Conversely values for SOD activity, which is inversely proportional to the degree of cytochrome c oxidation over time, showed significantly higher activity during the July full moon cycle versus collection periods throughout the sampling cycle (p = 0.0454 – 0.0002, CI = 95%, figure 13). This would set SOD activity as being highest following the reproductive peak of *P. damicornis*. Acute sampling analysis displayed day 5 of the August ¼ moon as having significantly higher SOD activity versus days 3 and 4 (p = 0.0351 and p = 0.0004, respectively, CI = 95%, figure 14). However, day 5 SOD activity was not significantly different than measured activity from day 1 and day 2 (p > 0.05, CI = 95%). What is more, day 2 activity was significantly higher than that calculated during day 4 (p = 0.0224, CI = 95%), suggesting that SOD activity dropped significantly before increasing again between moon phases.

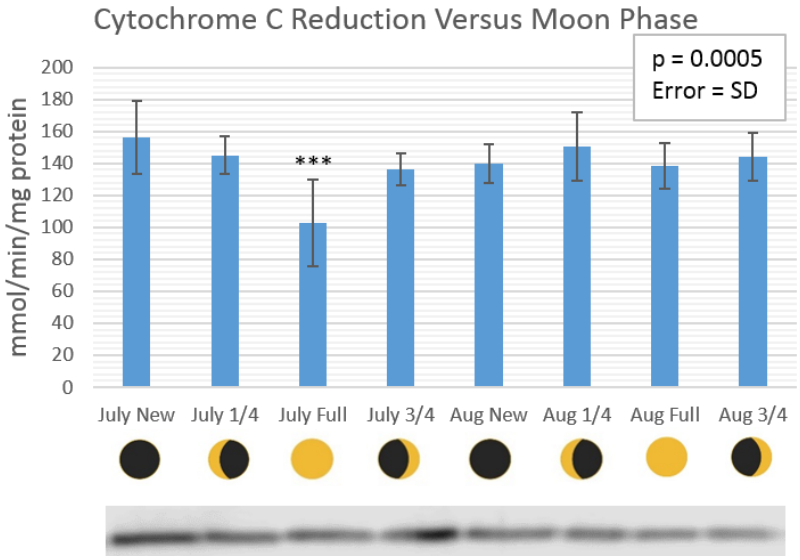


Figure 13. Superoxide dismutase activity characterized by inhibition of cytochrome c reduction mmol/min/mg protein versus bleaching cycle; lower cytochrome c metabolism correlating to higher SOD activity. Activity of SOD was significantly higher than July new and ¼ moon phases; bars represent mean ± SD (p = 0.0005, CI = 95%).

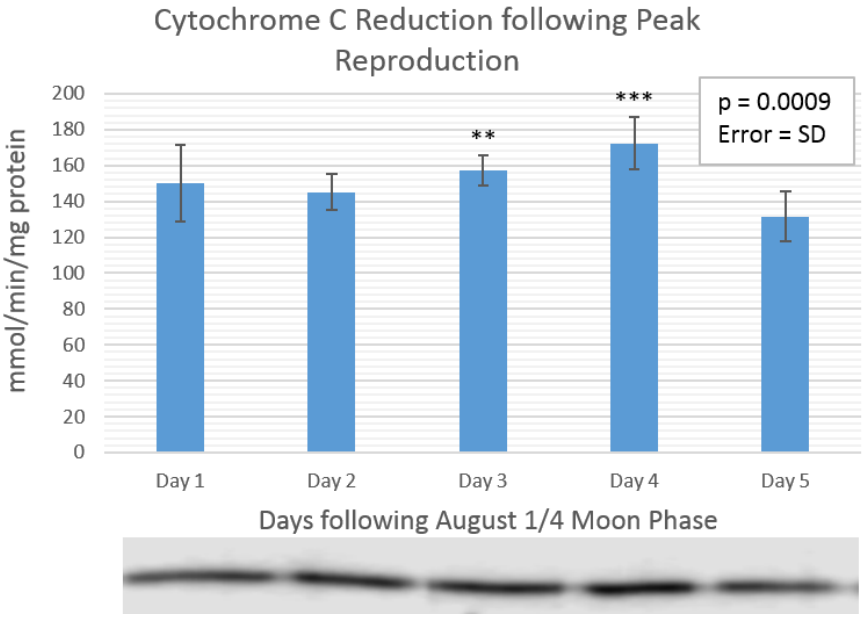


Figure 14. Acute tracking of SOD activity characterized by the inhibition of cytochrome c reduction mmol/min/mg protein versus time following peak reproduction (¼ moon phase); bars represent mean ± SD (p = 0.0009, CI = 95%). Samples collected during day 1 of the ¼ moon phase were found significantly lower in activity versus those samples on day 3 (p = 0.031).

Western Immunoblotting

Western blots evaluating for the presence of this suite of enzymes in coral samples confirmed the presence of SOD throughout time points, though band intensity did not appear to qualitatively vary between datasets (figure 13 and 14). These results were not replicated for the additional three enzymes of interest. Due to batch-to-batch variability in the polyclonal antibodies employed to evaluate CAT, GR, and GPx presence/absence in coral samples, chosen primary antibodies that worked in *P. damicornis* samples during pilot analyses did not work during final Western blot protein analyses.

Potential Enzymatic Indicators of Coral Bleaching Induced by Heat Stress

Enzymatic Activity Assays

The assay for the CAT enzyme suggests that CAT activity significantly increased between pre-bleaching and bleaching levels ($p = 0.0026$, CI = 95%), before significantly dropping again from bleaching to recovery ($p = 0.0005$, CI = 95%, figure 15). To note, pre-bleaching values for CAT activity were not significantly different than CAT activity for recovery samples ($p > 0.05$, CI = 95%). Values for CAT activity during bleaching (5009 ± 592.1 mmol/min/mg protein) were about 1.5 times higher than measured CAT activity for pre-bleaching and post-bleaching samples (3431 ± 543.8 and 3019 ± 112.6 mmol/min/mg protein, respectively).

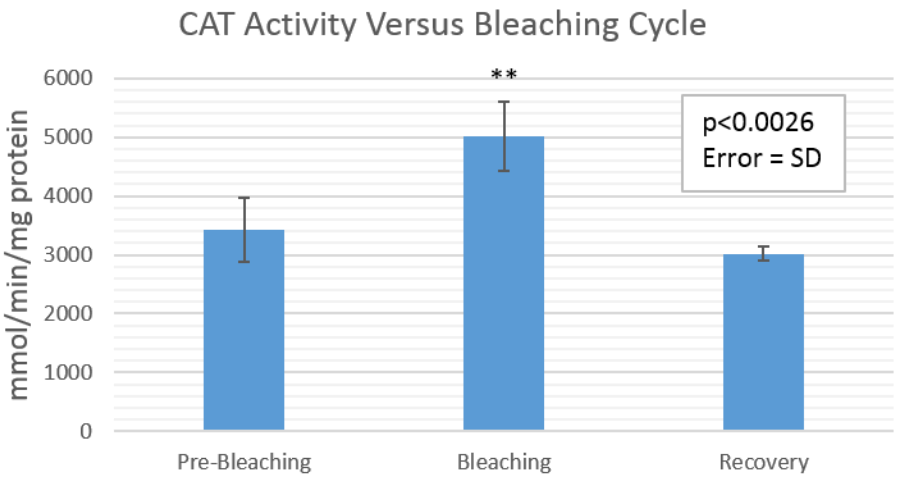


Figure 15. Catalase activity in mmol of H_2O_2 metabolized/min/mg protein versus bleaching cycle; bars represent mean ± SD. Bleaching samples expressed significantly higher activity than pre-bleaching and recovery samples ($p < 0.0026$, CI = 95%).

Conversely, GR (figure 16), SOD (figure 17), and both Se-dependent and independent GPx (figures 18 and 19, respectively) activities did not significantly vary from pre- to during to post-bleaching levels ($p > 0.05$, CI=95%). Though GR showed small trends in minimum activity occurring during the bleaching event, the variation in activity was not significant. Likewise, peak inhibition of cytochrome c reduction by SOD appears to have peaked with bleaching, this trend was not significantly different from pre- and post-bleaching samples.

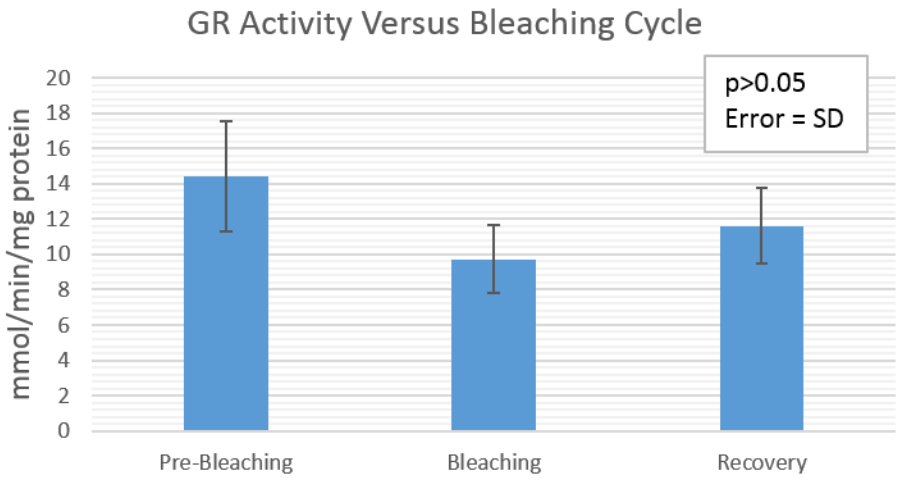


Figure 16. Glutathione reductase activity in mmol of NADPH metabolized/min/mg protein versus bleaching cycle; bars represent mean ± SD ($p > 0.05$, CI = 95%).

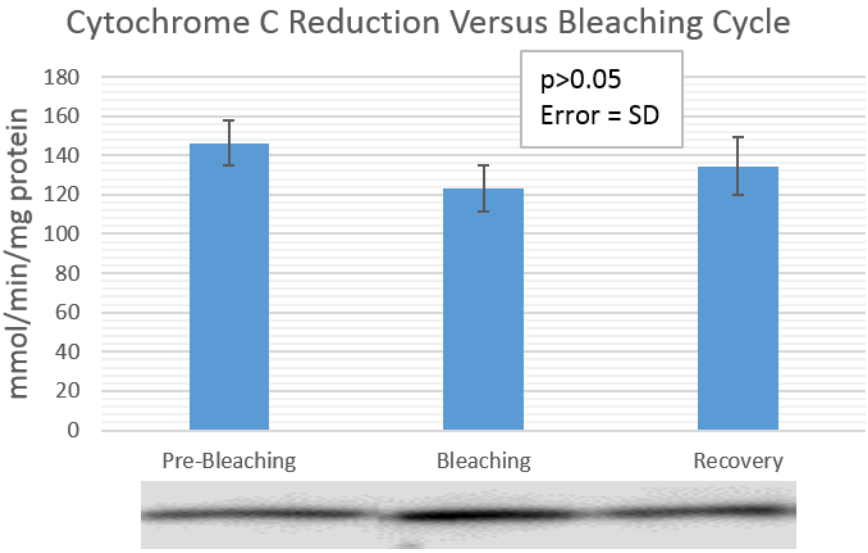


Figure 17. Superoxide dismutase activity characterized by inhibition of cytochrome c reduction mmol/min/mg protein versus bleaching cycle; lower cytochrome c metabolism correlating to higher SOD activity. Bars represent mean ± SD ($p > 0.05$, CI = 95%). Additional western blots confirming SOD-1 enzyme presence versus bleaching cycle display slightly darker banding for bleaching samples, qualitatively suggesting higher concentrations of SOD-1 in bleached coral tissues. To facilitate visual comparison of Western blot data with enzyme activity, the Western blot image was cropped to remove extraneous samples included from reproductive cycling investigation data. Full images are included in appendix.

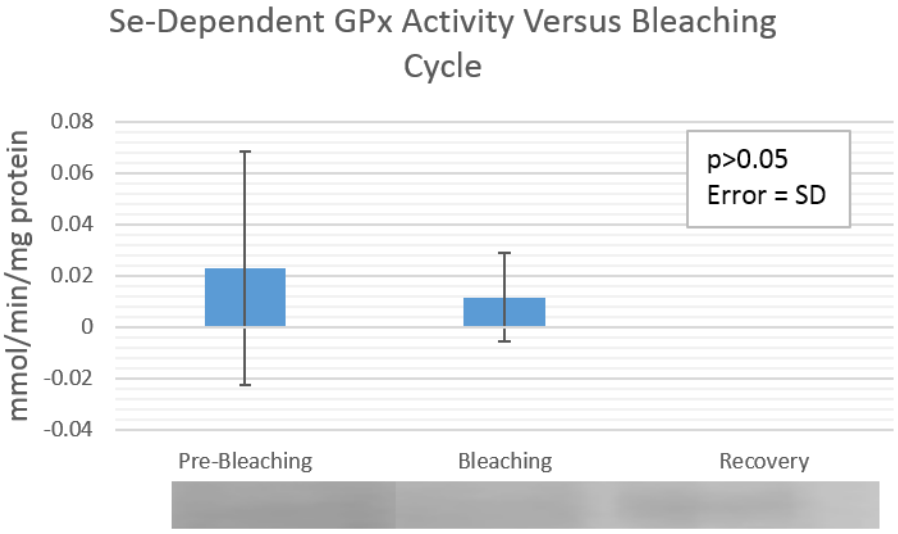


Figure 18. Se-dependent GPx activity characterized by the consumption of NADPH mmol/min/mg protein versus bleaching cycle; bars represent mean ± SD ($p > 0.05$, CI = 95%). Additional western blots confirming GPx-1 enzyme presence versus bleaching cycle. Banding is extremely light, which corresponds to little to no observed GPx activity. To facilitate visual comparison of Western blot data with enzyme activity, the Western blot image was cropped to remove extraneous samples included from reproductive cycling investigation data. Full images are included in appendix.

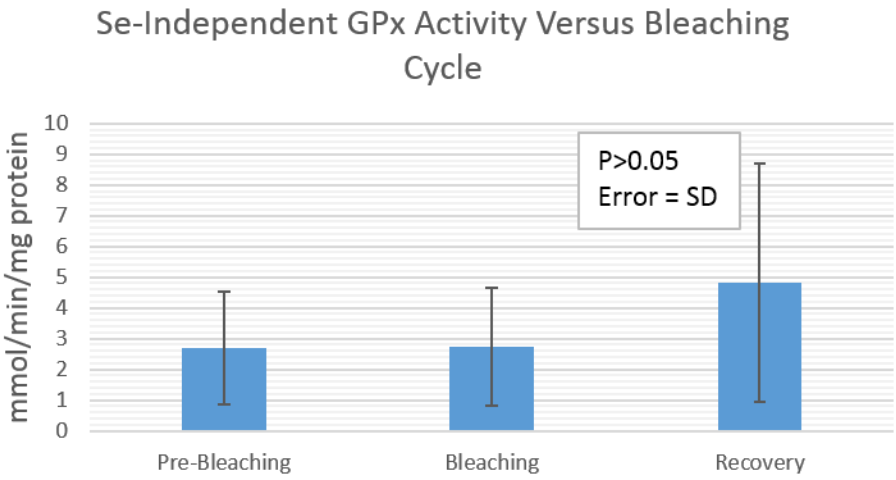


Figure 19. Se-Independent GPx activity characterized by the consumption of NADPH mmol/min/mg protein versus bleaching cycle; bars represent mean ± SD ($p > 0.05$, CI = 95%).

Western Immunoblotting

Western blots evaluating the presence of these antioxidant enzymes in pre- to bleached to post-bleaching samples confirmed SOD presence in coral tissues (figure 17). Consistent with enzyme activity, the band for SOD during bleaching appeared marginally darker than those for pre-bleaching and recovery. Bands for GPx-1 were barely visible following blotting, though this is consistent with enzyme assays, as Se-dependent GPx activity was negligible (figure 18). As was discussed in our investigation of the effect of reproductive cycling on antioxidant enzyme responses, these results were, again, not able to be replicated for CAT and GR, as new primary polyclonal antibodies for these enzymes did not bind to samples.

Role of Symbiodinium spp. Clade on Coral Health and Implications of Mass-Bleaching

Symbiont Variation Visualization

Comparisons of RFLPs between samples collected over a complete bleaching cycle displayed no variation in symbiont clade recruitment (figure 20). Had variation between clade recruitment been detected, banding differences would have been observed between the different time points. The results showed that symbiont-host assemblages were conserved over the full stress-recovery period.

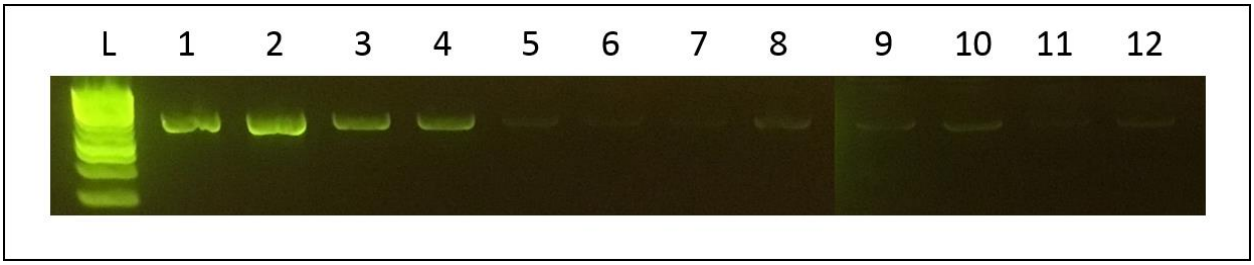


Figure 20. Agarose gel electrophoresis of DNA isolated from Symbiodinium associated with sampled P. damicornis colonies (primers ss3z, ss5z; GelGreen stain, 1 kB Plus DNA Ladder (Invitrogen)). Lanes 1-4 represent pre-bleached samples colonies 1, 2, 5, 6, respectively; lanes 5-8 represent bleached samples colonies 1, 2, 5, 6, respectively; and lanes 9-12 represent post-bleached samples colonies 1, 2, 5, 6, respectively. Due to limited lane number, samples described in wells 9-12 were run on a separate gel and the resulting image was spliced together for side-by-side comparison.

Chapter 5: General Discussion

Antioxidant Enzyme Cycling over Reproductive Lunar Cycles in *Pocillopora damicornis*

In order to develop rapid and efficient tools for detecting sub-lethal levels of stress in corals, first there is a need to define foundational changes in coral protein expression and activity patterns across normal homeostatic processes (Downs et al., 2012; Rougée et al., 2014). Without such definition, there is potential to mistake significant variations in coral health for responses to stress exposure, rather than those due in-part to normal biological processes based on the timing of coral sampling with respect to baseline (Rougée et al., 2006). For example, there resides great importance and versatility in the use of CAT, GR, SOD, and GPx in the definition of ROS-induced stress in coral animals, which aids in better defining such information as seasonal stress variations, xenobiotic impacts, and thermal stress limitations in corals (Downs & Downs, 2007; Griffin, Bhagooli, & Weil, 2006; Higuchi et al., 2008; Lesser, 1996; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010). As such, work to characterize potential basal levels of activity of these enzymes will benefit the coral conservation biology community and aid in improving experimental design by accounting for background levels of stress flux due to innate biological processes. Further, results from this study help bolster this effort to assess reproductive baselines in antioxidant enzyme capacity, as this study found activity values significantly varied in relation to reproductive cycling for CAT, GR, SOD, and GPx.

Similarly to those findings documented by Ramos, Bastidas, Debrot, & García (2011) in *S. siderea*, a significant peak in CAT activity was observed with relation to peak reproduction in *P. damicornis* (figure 5). Though acute sampling did not detect day-to-day changes in activity values following peak planulation in August (figure 6), August ¼ moon (peak reproductive output) CAT activity was significantly higher than that of July full moon (off-peak reproductive output) activity values ($p = 0.0177$, CI = 95%). Unfortunately, batch-to-batch variability in the specificity of polyclonal antibodies obscured any ability to evaluate for CAT presence through western blotting. Disappointingly, antibodies that were found to work in *P. damicornis* during the preliminary study data failed to work in corals the following year using fresh but different batches of antibodies. This issue is one arising from the lack of antibodies specifically

developed for targeting corals and relying upon cross-species reactivity in common epitopes, rather than a coral-targeted epitope. Additionally, inherent variations in batches during the development of polyclonal antibodies may have caused such issues in enzyme detection. As such, we can only state that these results suggest, rather than definitively confirm, catalase activity values significantly peak with relation to reproductive brooding output. However, by designing these assays to specifically cater toward conditions favoring optimal CAT activity, paired with evaluations of other H_2O_2 scavenging enzymes, such as Se-dependent GPx that found little to no H_2O_2 consumption, these results strongly suggest that CAT activity significantly increases under peak reproductive output. The implications of these also findings suggest that in accordance to what has been observed in other coral species and other organisms, reproduction is a process, during which endogenously generated levels of stressors (ROS) are produced in the coral *P. damicornis*, and consideration should be taken into account, with respect to reproductive cycling, when surveying and comparing coral populations for variability in antioxidant enzyme activity (Agarwal et al., 2005, 2006; Fujii et al., 2005; Ramos et al., 2011; Rougée et al., 2014).

The finding that significant increases in GR activity are associated with reproductive peaks ($p = 0.0001$, CI = 95%, figure 7) provides further evidence for the need to consider reproductive time points when using antioxidant enzymes as biomarkers for oxidative stress evaluation. In conjunction with these findings, results illustrating significant decreases in GR activity over the 5-day acute sampling period provide better resolution in identifying the rate at which antioxidant enzyme activities can significantly change over natural *P. damicornis* brooding cycles ($p = 0.0162$, CI = 95%, figure 8). Unfortunately, there again was no clear pattern observed through western blotting, as band intensity was poor in all samples, making relative quantification difficult. In order to overcome this consistent issue facing the detection of such enzymes through western immunoblotting, work must be done to design better antibodies in the future. Such work will improve our ability to examine expression of these biomarkers in additional studies examining oxidative stress response. Despite this, results of GR activity assays provide useful insight into the replenishment of the powerful antioxidant, reduced-glutathione, under reproductive pressures. These findings also suggest that under reproductive peaks,

enzymes are utilizing glutathione to reduce ROS to less reactive forms at a more rapid pace. Such evidence would propose that enzymes, such as GPx would display peaks in activity during reproduction, accordingly. However, this was not observed in sampled colonies.

Interestingly, and inverse to what was expected in response to potential increases in ROS defenses during reproduction, GPx displayed significantly greater activity during full and ¾ moon, rather than new and ¼ moon phases (figure 11). Greater, Se-dependent GPx was not significantly active, as activity assays found little to no detectable activity during the full study period (figures 9 and 10). Further, in western blots examining Se-dependent GPx-1, bands were again poorly defined, suggesting that either Se-dependent GPx is not expressed in *P. damicornis* or that the chosen antibodies failed to bind to GPx-1 residues. This is also interesting, as preliminary pilot work utilizing heavily stressed *P. damicornis* samples displayed defined bands relating to GPx-1 presence. It is possible that associated ROS production during reproductive peaks may not be enough to elicit synthesis of GPx, or that enzymes, such as CAT are favored as primary responders to low-levels of ROS within coral tissues. However, studies analyzing GPx activity during stress exposure and seasonal changes in corals belonging to the genus *Pocillopora* confirmed GPx activity and presence (Downs et al., 2012; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010; Vijayavel et al., 2012). Rather, observed GPx-related activity was detected for Se-independent GPx isozymes, with significant increases in activity during the July ¾ and full, and August ¾ moon phases (figure 11). Additional analysis of acute changes to Se-independent GPx activity following peak reproduction during the ¼ moon phase found a significant increase in activity during the third day following the ¼ moon (figure 12). Though activity was not found to be significantly higher than that of the August ¼ moon until the subsequent ¾ moon cycle, this suggests that there may be significant increases in Se-independent GPx activity detectable directly following reproductive peaks and increasing over the succeeding two weeks. These findings are perplexing, as activities inversely mirror those expressed in both CAT and GR. Further, it would be expected that observed significant increases in GR activity would be directly proportional to that of GPx enzymes, as activity in GPx depletes reduced glutathione pools and continued activity requires replenishment of this co-factor.

Although enzyme assays were specifically designed to function with respect to *P. damicornis* samples, as a result of these findings, it would be advisable that Se-dependent GPx activity assays be reconsidered and redesigned to better evaluate Se-dependent GPx activity, specifically. Additionally, while GPx-1 is a widely expressed GPx isozyme commonly located within cellular cytosol, mitochondria, and nucleus, it is possible that corals employ different forms of this enzyme as an adaptive function of their cellular detoxification (Margis, Dunand, Teixeira, & Margis-Pinheiro, 2008). Such variation in isozyme utilization has been previously described in studies of other marine invertebrates, including corals, with respect to their response to hypoxia and anaerobic respiration (Eberlee, Storey, & Storey, 1983; Fields, 1983; Fields, Eng, Ramsden, Hochachka, & Weinstein, 1980; Murphy & Richmond, 2016; Plaxton & Storey, 1982). Our findings for Se-independent GPx peak activity, are supported by the work of Rougée, Richmond, & Collier (2014), wherein the antioxidant enzyme glutathione-s-transferase, which also utilizes reduced glutathione for pro-oxidant detoxification, and the UDP-glucuronosyltransferase family expressed significantly higher activity 2 weeks following planulation in *P. damicornis*, rather than during peak planula release.

Building upon trends observed for GPx activity and additional antioxidant enzymes analyzed by Rougée, Richmond, & Collier (2014), SOD activity was found to retain a small, but significant increase during the July full moon phase, 1 week following peak reproduction ($p = 0.0005$, CI = 95%, figure 2.13). Acute sampling following the August ¼ moon also found activity to significantly vary, with days 3 and 4 displaying significantly lower SOD activity than during day 5 ($p = 0.0009$, CI = 95%, figure 2.14). However, these findings do not aid in illustrating significant trends in SOD activity following reproductive cycles in *P. damicornis*. Rather, this variation highlights potential acute day-to-day shifts in SOD activity unrelated to reproductive cycling. Supplementary investigations of daily shifts in SOD activity over monthly cycles would help clarifying if detected significant decreases in SOD activity during days 3 and 4 were attributable to slight variations in environmental conditions during sampling, or if SOD activity consistently experiences repeated significant declines in the days following reproductive peaks. In contrast to Western immunoblotting results for CAT, GR, and GPx, Western blots for SOD confirmed enzyme presence across all sampling points (figure 2.13). These

aids in validating observed activities of SOD obtained through enzyme assays and provided confirmation that assays were effectively designed to evaluate for activity in these samples. Although significant variations in SOD activity were not observed across the rest of the sampling periods, aside from the July ¾ moon, these findings suggest that SOD activity should be considered when accounting for oxidative stress responses during reproductively active periods in these corals.

These results highlight avenues for continuing studies, as greater investigation into the interplay of ROS generation and detoxification during coral reproduction allude to significant inherent stress thresholds in corals. These results also aid us in addressing the two of the hypotheses we sought out to test. With regard to hypothesis 1, we were able to reject the null that there would be no significant changes in enzyme activity over reproductive cycles. In testing hypothesis 2, challenges in assessing enzyme presence through Western blotting led to failures in our ability to challenge our null hypothesis. However, with respect to SOD, we can accept the null hypothesis, as SOD presence was both detected in all coral samples, regardless of reproductive phase and qualitative observations of banding do not yield any appreciable variations in the presence of SOD within samples (figure 2.13). As such, these thresholds and definitions of baseline expression levels help inform us of the specific collection periods needed to factor in biological processes naturally cycling in corals, before assigning an external stressor as the causative agent (Ramos et al., 2011; Rougée et al., 2014). Now that these data exist, demonstrating that coral in the field may be undergoing cyclical variations in enzyme activity with relation to reproduction, there is a need to replicate these results in a controlled laboratory setting. This will allow us to fully control for potential environmental pressures. Although environmental factors were controlled as much as possible with regards to collection times, tides, and sampling around weather anomalies, additional validation of these findings in a controlled setting, such as growth tanks, would be a natural progression with respect to this research. This work, however, also highlights deficiencies in our current capacity to expand our examination of coral proteomics, as limitations exist in our capability to achieve confirmation of protein presence through Western blotting. Due to inherent flaws in the reliability of polyclonal antibodies with respect to batch-to-batch variations in specificity, future work developing monoclonal

antibodies specific for enzymes, such as CAT, GR, and GPx, would build greater confidence in associated enzyme kinetic assays. This would allow for greater flexibility to diagnose stress biomarkers through enzyme assays, Western blotting, and enzyme-linked immunosorbent assays (ELISAs) that would aid in quantitatively evaluating enzyme concentration variation across sampling time points.

Understanding the influence of reproduction on various biomarker enzymes in coral remains a poorly characterized field that merits further expansion. The enzymes employed in this study have been widely applied to evaluate the effect of many a/biotic stressors on coral health (Flores-Ramírez & Liñán-Cabello, 2007; Higuchi et al., 2008; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010; Richier et al., 2003; Verma, Mehta, & Srivastava, 2007; Yakovleva, Bhagooli, Takemura, & Hidaka, 2004). Yet, refraining to consider reproduction as a source of inherent significant levels of ROS-induced stress responses presents a potential vulnerability in studies utilizing these biomarkers for stress detection by mistakenly characterizing natural fluctuations in antioxidant biomarkers for stress responses.

Potential Enzymatic Indicators of Coral Bleaching Induced by Heat Stress

Significantly higher CAT activity during the height of bleaching is consistent with findings in other corals displaying peak CAT activity during heightened thermal exposure (Higuchi, Fujimura, Arakaki, & Oomori, 2008; Higuchi, Yuyama, & Nakamura, 2015; Liñán-Cabello et al., 2010)(figure 15). This suggests that in response to increased production of H_2O_2 through heightened thermal exposure (Asada, 2006; Gates et al., 1992), CAT activity has been upregulated to compensate for increased ROS proliferation. Interestingly, CAT activity did not significantly drop over summer to winter cycles (pre-bleaching to recovery), as was documented in the seasonal variability of the coral *P. capitata* (Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010). This may be due to the intensity of the heating anomaly in 2014, standing as one of the warmest years on record (Bahr et al., 2015). Extended recovery due to the duration of bleaching, in addition to the shallow, urban, coastal distribution of these corals could explain the lack of significant seasonal variation in CAT activity. Interestingly, unlike CAT, GR, SOD, and

GPx did not exhibit significant variations in enzyme activity from pre-, to during, to post-bleaching states.

It was originally anticipated that the highest activity of CAT, SOD, GPx, and GR would be detected during bleaching. However, these findings suggest that SOD, GPx, and GR activities may not increase with bleaching stress in *P. damicornis*. Potential significant increases in the activity of these enzymes may have peaked prior to bleaching, as removal of the primary source of ROS generation during the bleaching process (Asada, 2006; Gates et al., 1992) has the potential to have decreased the prevalence of O_2^- and H_2O_2 within coral host tissues.

Despite support by the papers cited above, our results do not reflect all other reports in the literature. Several studies have described significant increases, specifically of SOD, with increasing heat and subsequent bleaching stress (Flores-Ramírez & Liñán-Cabello, 2007; Higuchi et al., 2008; Lesser, Stochaj, Tapley, & Shick, 1990). With respect to these discrepancies, and with further regard to Figure 17, a trend in both the activity and potential concentration of SOD within sample tissues, such that they are higher during bleaching than during pre- and post-stress time points, was observed. This would suggest that sampling just prior to bleaching may elicit the hypothesized response from these enzymes, and/or that greater sample numbers might match these previously published data. Reports that have investigated the effect of thermal stress-derived oxidative damage on *P. verrucosa* health seem to confirm this (Rodriguez-Troncoso et al., 2013). These published studies demonstrate that SOD activity reaches maximum levels just prior to the expulsion of *Symbiodinium* from the coral (Rodriguez-Troncoso et al., 2013). If able to predict the time points just prior to colony bleaching, potential maxima for SOD, GR, and GPx may have been observed (Ainsworth et al., 2008). Further, by recognizing that CAT activity peaked during bleaching stress, it is possible that CAT functions as a more efficient scavenger of peroxides than the coupled system of GPx and GR in this organism. As a result: the lack of significant activity over the bleaching cycle in GR and both forms of GPx (figures 16, 18, and 19, respectively) might be attributed to significantly higher levels of CAT scavenging. Notwithstanding, these antioxidant enzymes may prove useful in the detection of oxidative stress caused by other

means and should be considered as useful biomarkers for understanding variability in coral molecular responses to stressors.

Although the primary antibodies for these specific enzymes were tested and confirmed to bind to all enzymes of interest during pilot work, their failure to bind to their target proteins during the main study highlights a textbook issue of polyclonal antibody use; namely: batch-to-batch variability. In order to circumvent this issue, monoclonal antibodies for CAT and GR would be recommended for use in the species *P. damicornis*, as these would ensure homogeneity across batch production and provide better resolution when attempting to quantify protein levels. Despite this recommendation, cost remains the limiting factor for generating and using such antibodies in this non-model organism. Moreover, while GPx-1 is the most common form of GPx and the form of which this work examined, further analysis examining other isozymes of GPx would aid in improving comprehensive investigations of coral health. Of the 7 currently described isozymes, 5 are categorized as selenium-dependent (GPx-1, 2, 3, 4, 6), while two lack selenocysteine in their active sites (GPx-5 and 7)(Margis et al., 2008). Further, compartmentalization of these isozymes within different cellular locations, specific tissues, and organisms, lends additional specializations towards their targeted function (table 6). There are several additional isozymes specific to plant and fungi; however, description of these remaining GPx classes require further classification and description (Margis et al., 2008). It has been previously documented that corals do not necessarily rely upon the same enzymes for metabolic functions seen in other model systems (Murphy & Richmond, 2016). Therefore, consideration should be given to targeting alternate forms of GPx may provide more information on the nature of this enzyme's expression. This is especially true, as Se-dependent GPx expressed little to no activity during the study period, whilst Se-independent GPx, which metabolizes organic forms of peroxides, displayed enzyme activities several magnitudes higher than its Se-dependent counterpart.

Table 6. GPx isozyme classification and localization.

GPx Isozyme	Cellular Compartmentalization
GPx-1	Cytosol, nucleus, mitochondria
GPx-2	Cytosol and nucleus
GPx-3	Cytosol
GPx-4	Nucleus, cytosol, mitochondria, cellular membranes
GPx-5	Mammalian epididymis
GPx-6	Human and pig olfactory epithelium
GPx-7	Mammalian tissues

Similar to the systems used for humans, the methods of health evaluation change with respect to underlying pathology of the disease(s). By building a suite of biomarkers from which to draw information about coral stress responses, the ability to predict coral health analyses will achieve better diagnostic power. The results of this specific study function as a platform to expand our future work. With respect to this: future investigations will seek to refine the resolution of this work by

1) using this model of long-term heat stress, with ramping and recovery in a lab setting, and
2) increasing sampling to include fine-scale modeling of acute changes to coral health leading up to bleaching and following the apex of thermal stress.

It will be necessary to incorporate a large pool of samples in this survey, expanding sampling to various *P. damicornis* habitats, as findings studying the activity of SOD and CAT in *Siderastrea siderea* found that antioxidant enzyme activity varied based on location (Ramos, Bastidas, Debrot, & García, 2011). This will aid the Scientific Community to better control for unpredictable global and local environmental factors that may modify coral stress responses, such as those identified herein. In addition, future use of fine-scale sampling will also assist other researchers to understand modifications in enzymatic responses to pre-bleaching stress and provide insight into the rate at which recovery is measurable following the removal of temperature as a source of stress in *P. damicornis*.

The demonstrated and discussed expansion of molecular tools for evaluating stress in *P. damicornis,* which have been made available and validated (by us) are aimed

toward meeting a critical need to detect coral reef stress prior to extreme physical manifestations, such a death/bleaching. Serious concerns have been, and continue to be, expressed by acknowledged experts across the global coral community, because extreme thermal events are becoming more common, and corals are becoming more vulnerable to catastrophic events, including anthropogenic sources of stress (Bruno et al., 2007; Edge et al., 2013; Harvell et al., 1999; Sudek et al., 2015; van de Water, Lamb, Heron, van Oppen, & Willis, 2016; J. R. Ward et al., 2007). By investigating the molecular mechanisms of coral bleaching, and hopefully identifying these "checkpoints" before bleaching or death, we hope to provide the marine conservation and restoration communities better tools to identify stress pre-mortem and thereby adapt management strategies to anticipate bleaching events (van de Water et al., 2016). These results provide additional insight for reef managers and those studying oxidative stress in corals on thresholds for *P. damicornis* enzymatic responses to thermally-induced bleaching pressures and guidance toward curating collections as to better evaluate peak stress response prior to bleaching. By adapting these lessons into future practices, improvements can be made to early stress detection and potential mitigation and/or improved monitoring efforts.

Role of Symbiodinium spp. Clade on Coral Health and Implications of Mass-Bleaching

It is well-documented that the symbioses between corals and *Symbiodinium* spp. provide coral hosts with varied levels of benefits and costs, based on clade associations (Cunning et al., 2016; Cunning, Yost, Guarinello, Putnam, & Gates, 2015; Haryanti, Yasuda, Harii, & Hidaka, 2015; Rands et al., 1992; Rowan et al., 1997; Silverstein et al., 2015; Stat et al., 2008; West & Salm, 2003). Rapid changes to environmental norms can lead to the disassociation of coral-symbiont symbioses, resulting in coral bleaching and potential long-term health consequences if not quickly reversed (Glynn, 1993; Lesser, 1997). Instances of global thermal-stress events, causing bleaching and wide-spread coral mortality, is on the rise and is predicted to continue to increase in frequency (Eakin et al., 2016; Heron et al., 2016; Hughes et al., 2017). The global climate is undergoing record year-to-year temperature increases, further accelerating potential impacts to environmental deterioration of the marine tropical ecosystems in which corals reside

(Hughes et al., 2017). Furthermore, atmospheric CO_2 levels have also reached record levels and will not level off and decrease unless significant changes are made to reduce the production of greenhouse gases (World Meteorological Organization, 2017). As such, understanding how zooxanthellate-coral symbiosis influences their adaptive capacity to environmental changes has become ever more so important.

Studies have shown that some corals have the ability to shuffle their symbiont assemblages to enhance thermal resilience (Cunning, Silverstein, et al., 2015; Jones, Berkelmans, van Oppen, Mieog, & Sinclair, 2008; Silverstein et al., 2015), but the *P. damicornis* symbiont RFLPs in this experiment identified no detectable *Symbiodinium* clade shifts over the cycle of a natural heat-induced bleaching and recovery (Figure 20); thus the null hypothesis (hypothesis 5) cannot be rejected. The results here are consistent with the responses of other species in the genus *Pocillopora* in the eastern Pacific, as well as the rice coral *M. capitata* from Kāneʻohe Bay during the 2014 bleaching event (Cunning et al., 2016; McGinley et al., 2012); these corals did not shift their symbiont associations. Yet, gradients of partnerships with different symbiont clades can exist in these species (Rowan et al., 1997). Examining pre- and post-bleaching symbiont clade dominance in eastern Pacific *Pocillopora* spp. revealed that Pocilloporids can partner with both thermo-tolerant and sensitive clades, and greater proportions of sampled bleaching-resistant colonies contained clade D (Glynn et al., 2001). Further, variable susceptibility to bleaching in *M. capitata* during the 2014 heating anomaly in Hawaiʻi was attributed to corals harboring clade C and clade D at varying proportions (Cunning et al., 2016). Although partnerships with resistant *Symbiodinium* clades could afford greater bleaching resistance under extreme heat events (Silverstein et al., 2017), such evidence suggests that other factors may influence the conservation of associated and/or dominant symbiont lineages within these colonies.

While there were no detectable shifts in the associated symbiont clade for the *P. damicornis* sampled in this study, it is also important to recognize that the 2014 bleaching event was the first of four recent, consecutive bleaching events in the Hawaiian Islands (Eakin et al., 2016; Heron et al., 2016). Work by Cunning, Silverstein, and Baker (2015) suggests that the adaptive capacity to shuffle symbiont communities is tied with respect to severity of bleaching and duration of warming into the recovery period. In colonies of

Orbicella faveolata, those subjected to low and medium bleaching severity (7-10 days of heat exposure) were found to re-recruit the less thermo-tolerant clade B following bleaching (Cunning, Silverstein, et al., 2015). Interestingly, those exposed to low and medium thermal stress who predominantly harbored clade D before experimental heat exposure shifted clade dominance towards clade B post-bleaching (Cunning, Silverstein, et al., 2015). Only in those corals that experienced extended thermal exposure for 14 days, followed by higher recovery temperature (29°C versus 24°C), performed clade shuffling towards thermo-tolerant clade D (Cunning, Silverstein, et al., 2015). As such, although the colonies monitored in our study did not exhibit clade shuffling during the 2014 bleaching cycle, recurrent or more severe bleaching events may drive corals that were previously shown to conserve associated clades, to shuffle symbionts. In addition to recognizing that certain thermal limits and elongated exposure to heat-stress may have significant effects on the recruitment strategies of corals, supplementary knowledge also provides greater insight into those strategies favoring pre-bleaching clade retention.

Recent calculations suggest that, with respect to thermal stress, coral hosts retain a low adaptive capacity towards gaining thermal resistance over multigenerational time scales (Császár, Ralph, Frankham, Berkelmans, & van Oppen, 2010; Sammarco & Strychar, 2009). These findings likely vary between coral species, however, low heritability of traits conferring adaptive increases in thermal tolerances – i.e. inherent increased expression of specific genes related to antioxidant defenses – indicate that the host thermo-acclimation does not translate well into thermo-adaptation on a greater scale (Császár et al., 2010; Sammarco & Strychar, 2009). Rather, rapid thermo-adaptation may be a critical and valuable trait that zooxanthellae possess (Sammarco & Strychar, 2009). Further, it was found that regardless of their inherent tolerances, both the thermally-resistant *Symbiodinium* clade D and more sensitive clade C shared the ability to adapt to increasing heat stress (Császár et al., 2010). Significantly increased heritability of characteristics, such as increased photosynthetic yield, improved cycling of the antioxidant xanthophyll, and increased production of photo-protective pigments under thermal stress over multiple generations reported in *Symbiodinium* can enhance their resilience and reduce bleaching pressures for host corals (Császár et al., 2010). Rapid adaptations in symbionts due to short generation cycles, spanning from one day to two

weeks, could offer greater thermal defenses to coral hosts without requiring clade shifts following bleaching events (Sammarco & Strychar, 2009; Wilkerson, Kobayashi, & Muscatine, 1988).

With increasing instances of thermal stress events (Hughes et al., 2017), continual adaptation of zooxanthellae over time may hold a key to lower the severity of sequentially occurring heat-stress events. Since *P. damicornis* showed no detectable shift in symbiont over the studied bleaching cycle, it may be that the associated clade of *Symbiodinium* already had some ability to protect themselves and hosts from photosynthetically generated ROS output and/or improved ROS suppression. These qualities may present the existing relationship as more beneficial for the fast-growing, generally shallow-water *P. damicornis* host (Hoeksema et al., 2014), rather than shifting to a more thermally tolerant symbiont, such as clade D, that does not provide similar photosynthetic yields. Further, as a brooding species that vertically transmits symbionts to their planula larvae (symbionts from parent colonies directly pass to their progeny), conservation of specific lineages of symbionts may hold greater benefits for the fitness of *P. damicornis*, rather than abandoning those inherited from parent colonies (Lesser et al., 2013; Richmond & Jokiel, 1984). Moreover, although this species can shift symbiont clades, this may be only accomplished under longer scales of time, rather than rapidly swapping *Symbiodinium* clades following a single bleaching event (Glynn et al., 2001). Continued long-term monitoring and experimental analysis of variable genetic responses to thermal stress will be needed to elucidate the true mechanisms behind symbionts shuffling.

In order to survive future projections of increased thermal stress (Maynard et al., 2015), corals must acclimatize and adapt to meet new thermal norms. Thermal stress is unavoidable and higher thermal tolerances will be required in the future for survival across all coral habitats (Hughes et al., 2017). Though not all corals may take advantage of the breadth of functional diversity different symbiont-coral interrelationships provide (McGinley et al., 2012), symbionts aid corals in combatting a myriad of different environmental conditions and potential stressors (Little et al., 2004; Rands et al., 1992; Stat et al., 2008; Suggett, Warner, & Leggat, 2017). Though many questions remain with respect to illuminating the drivers behind symbiont associations and potential adaptations

towards stress tolerance, our findings provided further understanding on the behaviors of *P. damicornis* under naturally-occurring thermally-induced bleaching stress.

General Discussion

Global climate change is occurring and shifting thermal baselines will have severe consequences on the greater health and fitness of coral reefs throughout the world. This is exemplified in the severity and scope of prolonged thermal anomalies and resultant global bleaching and mortality of shallow water corals over the past decade (Hughes et al., 2017). The importance of healthy, reef-building corals to the integrity of tropical marine ecosystems, island population food resources, global commercial revenue sources, and coastal flood and erosion protection is well-documented (Bishop et al., 2011; Brander et al., 2007; Cesar et al., 2003; Cesar & Van Beukering, 2004; Hughes et al., 2017; Munday, Jones, Pratchett, & Williams, 2008; White, Vogt, & Arin, 2000). Losing such critical marine invertebrates has the potential to cost billions of dollars to the global economy, increase the severity of impacts from coastal storms, and accelerate habitat loss, facilitating associated declines in the organisms that rely on corals for shelter and food (Cesar et al., 2003; Munday et al., 2008). These concerns have heightened awareness of the need to develop diagnostic tools such as molecular biomarkers to aid coral researchers in better understanding corals on a sub-cellular level and sub-lethal changes in health that occur when exposed to stressors (Aswani et al., 2015; Bellantuono et al., 2012; Downs et al., 2012, 2000; Marshall & Schuttenberg, 2013). As such, this work was designed to more completely characterize:

1) baseline changes to biomarkers that detect stress responses in a major reef-building species,
2) biomarkers that specifically identify stress from heat-induced bleaching events, and
3) if this coral species retains the capacity to adapt towards increased bleaching resistance through variable symbiont clade mutualisms.

The understanding that corals cycle enzyme activity with relation to normal biological processes, specifically reproduction, is a recent development, identified and illustrated in the last decade (Ramos et al., 2011; Rougée et al., 2014). Although ecological studies have demonstrated the sensitivity of corals to various environmental pressures, understanding basal cycling of enzymatic profiles, especially during extremely energy-intensive processes such as reproduction, has high relevance towards improving the development of stress detection indicators in corals and reducing potential for mistakenly characterizing natural cycling of biomarkers, such as antioxidant enzyme activity, for external stress exposure responses (Downs et al., 2012; Edge et al., 2013; Rougée et al., 2014). Further, while surveys analyzing bleaching severity and coral mortality have proved useful in characterizing the effect of stressors on coral reef health, these tools are more effective in damage evaluation rather than for supporting pre-mortem mitigation (Bahr et al., 2015; Jokiel & Coles, 1977; Martinez et al., 2012; Wolanski, Martinez, & Richmond, 2009). It is also well recognized that in addition to the importance of conducting work to characterize biomarkers for sub-lethal stress in corals, mutualistic algal symbionts that provide associated corals with benefits can be used to assess susceptibilities to environmental influences (Cunning et al., 2013; Lesser et al., 2013; McGinley et al., 2012; Silverstein et al., 2015, 2017; Stat et al., 2008). As such, tests were run to determine if coral bleaching following a thermal stress event had any influence on the zooxanthellae clade association with host *P. damicornis* colonies.

To achieve the goal of improving knowledge of coral molecular biology through the application of biomarkers for coral conservation, the studies encompassed in this dissertation have successfully characterized significant shifts in the activities of CAT, SOD, GPx, and GR in *P. damicornis* that suggests that these enzymes cycle naturally with monthly reproductive peaks and troughs (Jokiel, Ito, & Liu, 1985; Richmond & Jokiel, 1984). Our studies both support and are supported by findings from Ramos, Bastidas, Debrot, and García (2011) and Rougée, Richmond, and Collier (2014). The evidence presented herein describes inherent biological influences of stress on the activity of several enzymes useful in applications describing coral response to a myriad of stressors (Baker, 2014; Downs & Downs, 2007; Karthi, Sankari, & Shivakumar, 2014; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010; Verma et al., 2007). By

understanding the molecular implications of reproduction on coral health, this aids in accomplishing several things. First, the development of these bioassays for measuring CAT, SOD, GPx, and GR activity using 96-well microplates has improved analysis throughput efficiency and reduced technical error by eliminating the need to use cuvettes and increasing side-by-side replicate use. An added benefit of in-house assay development has also been reduced overall cost per assay, due to a shift away from expensive enzyme kinetic assay kits. Second, these results provide molecular confirmation of visual observations of tissue damage/ROS production and measurable impacts occurring during the reproductive process in a brooding coral. Brooding corals personally observed under a microscope have displayed tissue damage from planula larvae bursting forth through tentacles and tearing away from mesenterial filament attachments when leaving the parent colony. This damage, along with known regulation of pro/antioxidants during reproduction, had led to hypotheses that antioxidant defense enzymes were implicated in the brooding process (Agarwal et al., 2006; Rahal et al., 2014). Thirdly, our findings provide theoretical baseline references for CAT, SOD, GPx, and GR activity for future coral samplings. Of course, further attention must be paid towards defining potential shifts in baseline activity levels with respect to seasonality and colony location, as some colonies may express varying levels of activity with seasonal temperature shifts, proximity to watershed discharges, and distribution across reef isobaths (Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010; Ramos et al., 2011; Richmond et al., 2007; Wolanski et al., 2009). Finally, we have provided essential insights for coral biologists, and those promoting conservation and restoration efforts, for developing sampling methodologies to assess coral responses to different abiotic and biotic factors. Our contribution in developing, testing, and validating sampling protocols that instruct sample collections that take into account enzymatically relevant reproductive time periods, can enable other researchers to design studies with appropriate power, sensitivity and specificity for analyzing singular and combinatorial effects of impacts on coral health. This work provides a framework for benchmarking study outcomes to reproductive peaks (or otherwise) that can reduce or eliminate the errors incurred by reproductive enzyme cycling in previous studies that attempted to isolate the effects of specific sources of coral stress, independent of natural coral response.

The opportunity to field-test these antioxidant enzyme assays that describe and quantitate the impact of stressors on coral health conveniently arose in the weeks and months following the completion of sampling for the investigation of baseline shifts in antioxidant enzymes over reproductive cycles. We built upon the baseline data collected through mapping antioxidant enzyme activity shifts along reproductively-relevant moon phase cycles to provide insight into the shifts in activity for these same enzymes under naturally-occurring endobiotic as well as thermal stress events. This has provided a far more comprehensive characterization of coral health modulation under stress, than has previously been described, and has been especially useful when analyzing health changes in the same colonies previously characterized.

Although the results of the scientific investigations did not demonstrate significant shifts in the antioxidant enzyme activities of SOD, GPx, and GR, these data are informative. Moreover, the observations of significant increases in CAT activity during bleaching are likely significant.

We believe that, due in part to sampling tissues after corals had begun to bleach, we suspect that peak ROS stress and related increases in antioxidant enzyme activity (especially that of SOD, GPx, and GR) occurred prior to sample collection. This theory is supported by other research analyzing the impact of thermally-induced bleaching stress on coral health, which found significant increases in similar enzymes activity prior to the initiation of zooxanthellae expulsion (Rodriguez-Troncoso et al., 2013). Sampling just prior to bleaching, however, is difficult to accomplish in field settings because variable inter-colony thermal tolerances can introduce challenges for sampling bleached or bleaching colonies at the same time point. Further, rapid onset of bleaching can increase the challenges associated with pinpointing optimal sampling points unless long-term monitoring of colonies is carried out. With respect to the molecular analyses, collecting during peak stress at the start of bleaching, rather than after bleaching had occurred may have yielded different results. This being said, significant increases in CAT activity over the bleaching cycle did help further our understanding of how this coral responds to heat stress. This suggests that CAT may be more sensitive or important to ROS detoxification resulting from long-term thermal stress exposure. Yet, without continued and acute sampling, we cannot conclude that CAT retains the highest priority for ROS

detoxification, as bleaching may have simply reduced ROS load, such that SOD, GPx, and GR activities were not extraordinarily different than that resulting from pre- and post-stress exposure.

The opportunity to characterize *P. damicornis* responses to such impacts as those studied in our investigation of enzymatic responses to thermally-induced bleaching allowed for further investigation into whether *P. damicornis* employs symbiont shuffling, as other corals have been found to do, when acclimatizing for increased future stress resistance (Silverstein et al., 2015). Interestingly, *P. damicornis* colonies did not demonstrate variable mutualisms with other *Symbiodinium* spp. than the ones they harbored pre-bleaching. These results align with previously conducted studies analyzing *Pocillopora* spp. responses to bleaching in the eastern Pacific (Glynn et al., 2001; McGinley et al., 2012). Characterized among the fastest growing coral species throughout the eastern Pacific (*P. damicornis* has been documented to grow between 1.5 – 4.37 cm per year (Guzmán & Cortés, 1989; Richmond, 1987)), this disregard for shifting towards another zooxanthellae clade may be motivated by basal metabolic needs to support rapid growth (Little et al., 2004). Additionally, as a monthly brooding species, such lipid-rich processes may require mutualisms with symbionts that maximize energy production versus increasing thermal resistance. Alternatively, when paired with the findings from the investigation of potential symbiont shifts during bleaching stress, *P. damicornis* may simply be more reliant on host defense capabilities for stress reduction during thermal stress, rather than those afforded by the symbiont (Barshis et al., 2014).

Reef Management Application

The net outcomes of the research presented herein improve our ability to use molecular biomarkers as indicators of coral reef stress at the sub-lethal level, when intervention has the greatest chance of reducing mortality. These findings will also improve the breadth of our analyses when investigating the effects of point-source pollution on coral reef health by expanding our toolbox of molecular biomarkers for stress detection. Due to its wide distribution and well documented reproductive characteristics, *P. damicornis* has been shown to be a good model organism for studying the use of molecular biomarkers for diagnostics. Cohorts of larvae can be used to control

for genetic variation, and standardized techniques can be applied over a vast range, from the Red Sea to the eastern Pacific.

Future Directions

To build upon the work presented in this dissertation, and continue expanding knowledge concerning coral molecular biology, including a mandate to improve the needs coral reef conservation managers, there are several directions in which future research should be pursued. With respect to analyzing reproductive cycling of enzyme activity, this work would benefit greatly from mirrored studies replicating these analyses on *P. damicornis* in a laboratory setting; *e.g.* within controlled tanks wherein environmental factors can be removed and/or accounted for. Isolation of reproduction as the sole factor influencing significant shifts in the activities of CAT, SOD, GPx, and GR would further validate our findings from our investigation of the effect of reproduction on these enzymes. Moreover, laboratory isolation would aid future researchers in controlling for the effects of weather, tidal shifts, toxicant exposure, predation, thermal anomalies and other environmental factors to a much greater extent than what can be achieved in field analyses.

The results of these studies also raise additional questions as to whether similar shifts in these antioxidant enzymes can be detected in other coral species, and if so, the degree to which variable reproductive strategies can influence those species' stress profiles. Among the other major Hawaiian reef-building corals, *M. capitata* and *Porites* spp. could provide insight into the nature of CAT, SOD, GPx, and GR activity for broadcast spawning species on a seasonal scale (Neves, 2000; Padilla-Gamiño & Gates, 2012). Additional follow-up investigations targeting fine-scale monitoring of antioxidant enzyme activity over gradual thermal ramping, mimicking those characterized by the 2014 heating anomaly, would aid our research in better pinpointing how ROS load affects coral health leading up to bleaching and when peak stress can be detected.

Additional work should be attempted in order to develop correct, accurate, and specific primary antibodies for reliable detection of CAT, GPx, and GR across and amongst species. Also, antibodies suitable for SOD detection are needed, because the antibodies employed in this study and purchased from Santa-Cruz Biotechnology Inc.

(Dallas, TX, USA) have been discontinued, as have all Santa-Cruz polyclonal antibodies – a considerable problem for researchers using non-standard animal models [such as coral].. Through the recruitment of non-species-specific antibodies that have utility in corals, our research as well as future studies that investigate the effects of reproduction and thermal stress on endogenous coral responses, will gain rigor and provide further confirmation of our findings. In addition, it would be desirable to adopt ELISA technologies, allowing for comparisons of enzyme presence with activity value, providing understanding of whether corals are dedicating energy and resources towards increased synthesis of these defense enzymes over stress exposure periods.

Finally, although symbiont clade shuffling did not occur in *P. damicornis* over the bleaching exposure period, understanding that adaptation to stressors, such as thermal tolerance, has high heritability in zooxanthellae, suggests further questions of whether chosen clade mutualisms in *P. damicornis* have the potential to improve thermal tolerance over time (Császár et al., 2010). Although fundamental transcriptional differences exist across lineage-specific divides in zooxanthellae (Barshis et al., 2014), the potential for *Symbiodinium* spp. to adopt photosynthetic strategies to increase photoprotection, thereby reducing oxidative stress and photoinhibition, warrants further investigation of adaptation potential for *P. damicornis*-associated symbionts (Császár et al., 2010). Such studies will improve our understanding of whether those corals that do not shuffle symbionts instead harbor symbionts that promote inheritance of thermo-adaptive traits, making them less susceptible to bleaching in the future.

Summary and Final Conclusions

There is great need and urgency to develop and apply new technologies and approaches for stress evaluation, detection, and responses in corals. Leading researchers in marine sciences have discussed that fact that we are at a tipping point that cannot be ignored if reefs, in their current form, are to be preserved for the future (Hughes et al., 2017). Many in the field would venture to say that it may be too late to save the reefs known today and that local-scale management efforts will not be enough to effectively support conservation efforts (Hughes et al., 2017). However, in the face of new and emerging diagnostics such as we present here, even the most pessimistic estimations for

coral health should not cause us to lose hope. These studies have demonstrated physiological and endobiotic changes that can be used to formulate guidelines for sampling that can avoid confounding factors when utilizing antioxidant defense network enzymes. This is because we have, in some cases, identified inherent natural cycling of the enzymes in conjunction with reproductive time points. The outcomes from this body of work can improve the accuracy of our own, and others', research by seeking to employ sub-lethal stress indicators for use in coral tissue analyses. We also hoped that the outcomes from these studies will facilitate the integration of rapid, reliable molecular tools for coral health characterization in conservation and environmental impact studies.

The loss of coral reefs [globally] would severely cripple coastal marine ecosystems and handicap human services ranging from local food sources to the global economy (Bishop et al., 2011; Cesar, Burke, & Pet-Soede, 2003). As such, it is imperative that investigators continue to improve existing tools and introduce new tools/biomarkers for sub-lethal coral stress detection. We contend that the body of evidence presented makes a significant contribution to this imperative. It is also critical that entities supervising marine coastal conservation take-up and utilize newer methods, such as those presented here, to improve and inform their future management practices.

This research, and the future application of our findings regarding measurable and quantifiable determinants of coral reef health, can be implemented prior to catastrophic bleaching (death) events, can support local capacity for improving coral reef health, and facilitate a shift towards improved reef stewardship a global stage.

Appendices

E Hō Mai

E hō mai ka ʻike mai luna mai ē
ʻO nā mea huna noʻeau o nā mele ē
E hō mai, e hō mai, e hō mai ē

Give forth knowledge from above
Every little bit of wisdom contained in song
Give forth, give forth, oh give forth.

- Edith Kanakaole

Nā ʻAumākua

Nā ʻaumākua mai ka lā hiki a ka lā kau
Mai ka hoʻokuʻi a ka hālāwai
Nā ʻaumākua iā kahinakua, iā kāhinaʼalo
Iā kaʻa ʻākau i ka lani
ʻO kīhā i ka lani
ʻOwē i ka lani
Nūnulu i ka lani
Kāholo i ka lani
Eia na pulapula a ʻoukou ʻo ka poʻe Hawaiʻi
E mālama ʻoukou iā mākou
E ulu i ka lani
E ulu i ka honua
E ulu i ka paeʻāina ʻo Hawaiʻi
E hō mai i ka ʻike
E hō mai i ka ikaika
E hō mai i ke akamai
E hō mai i ka maopopo pono

E hō mai i ka ʻike pāpālua
E hō mai i ka mana.
ʻĀmama, ua noa

Ancestors from the rising to the setting sun
From the zenith to the horizon
Ancestors who stand at our back and front
You who stand at our right hand
A breathing in the heavens
An utterance in the heavens
A clear, ringing voice in the heavens
A voice reverberating in the heavens
Here are your descendants, the Hawaiians
Safeguard us
That we may flourish in the heavens
That we may flourish on earth
That we may flourish in the Hawaiian Islands
Grant us knowledge
Grant us strength
Grant us intelligence
Grant us understanding
Grant us insight
Grant us power
The prayer is lifted, it is free.

- David Malo

Oli Mahalo

‘Uhola ‘ia ka makaloa lā
Pū ‘ai i ke aloha ā
Kū ka‘i ‘ia ka hā loa lā
Pāwehi mai nā lehua
Mai ka ho‘oku‘i a ka hālāwai lā
Mahalo e Nā Akua
Mahalo e nā kūpuna lā, ‘eā
Mahalo me ke aloha lā
Mahalo me ke aloha lā

The makaloa mat has been unfurled
In love, (food is/was shared) we share
The great breath has been exchanged
Honored and adorned is the Lehua
From zenith to horizon
Gratitude and thanks to our Akua
Gratitude and thanks to our beloved ancestors
Gratitude, admiration, thanks, and love
To all who are present, both seen and unseen.

- Kēhau Camara

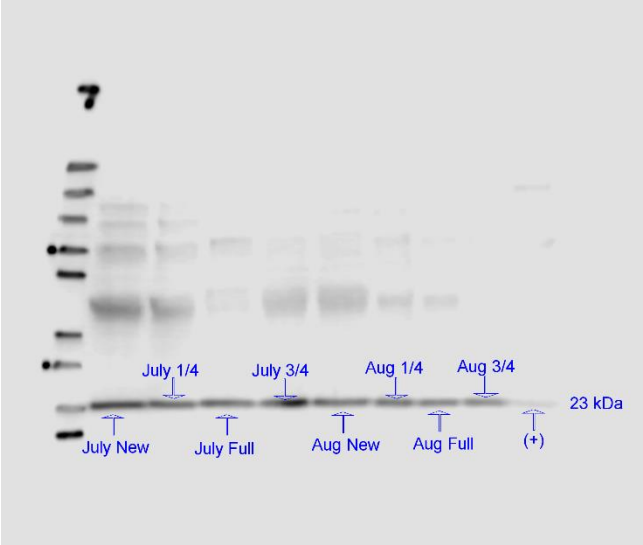


Figure 13. Full image.

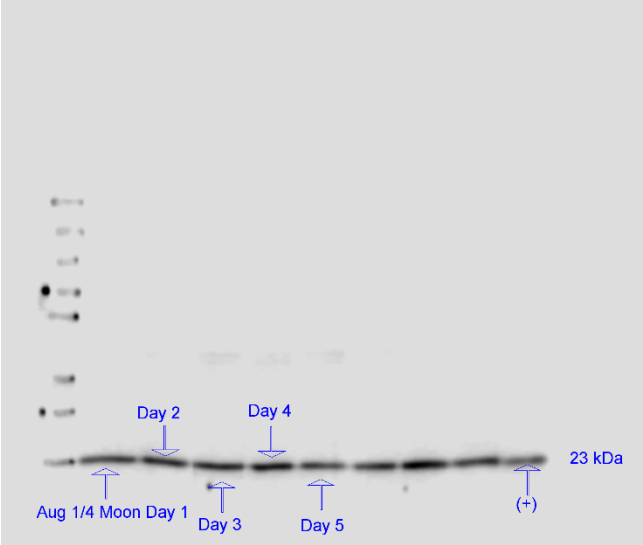


Figure 14. Full image.

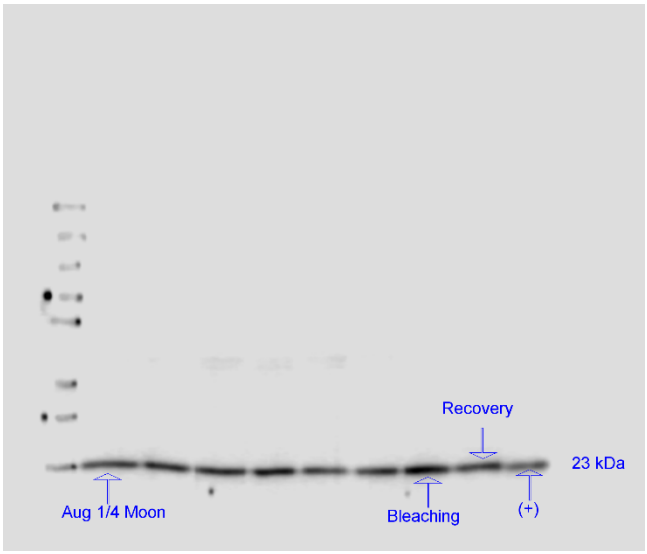
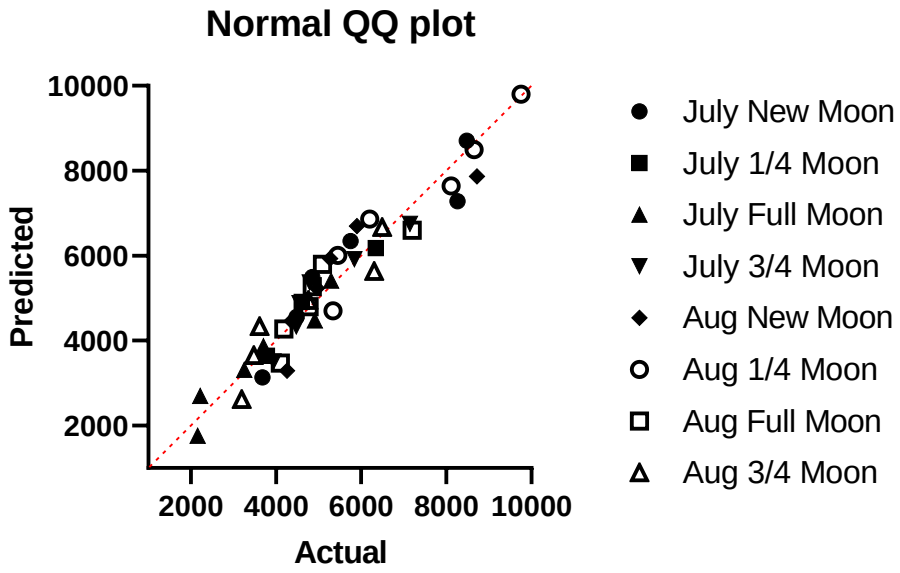


Figure 17. Full image.

Supplements

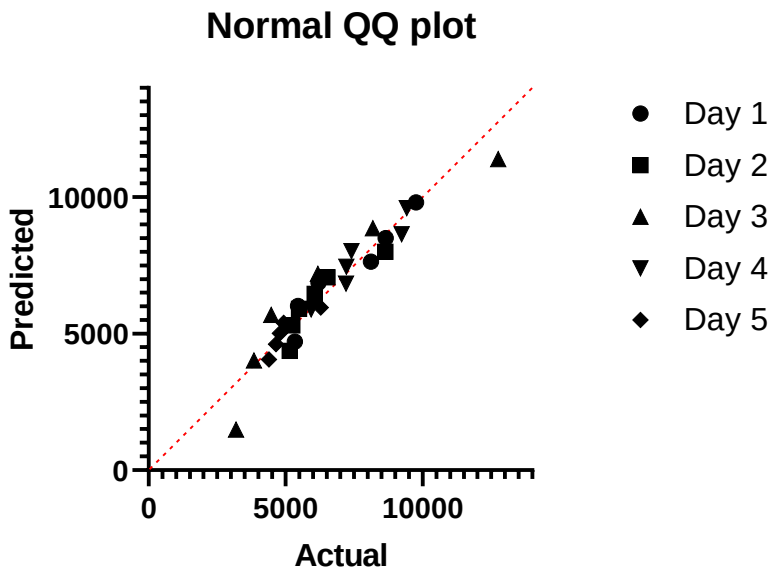
Supplements 1. D'Agostino and Pearson Omnibus normality test for catalase activity values over July and August reproductive timepoints.

D'Agostino & Pearson normality test	
K2	4.362
P value	0.1130
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



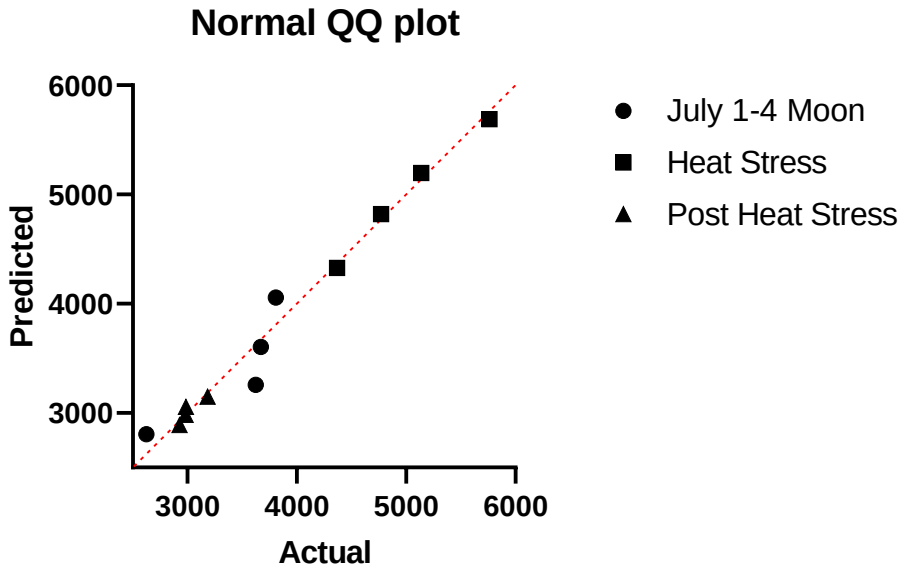
Supplements 2. D'Agostino and Pearson Omnibus normality test for catalase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

D'Agostino & Pearson normality test	
K2	4.585
P value	0.1010
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



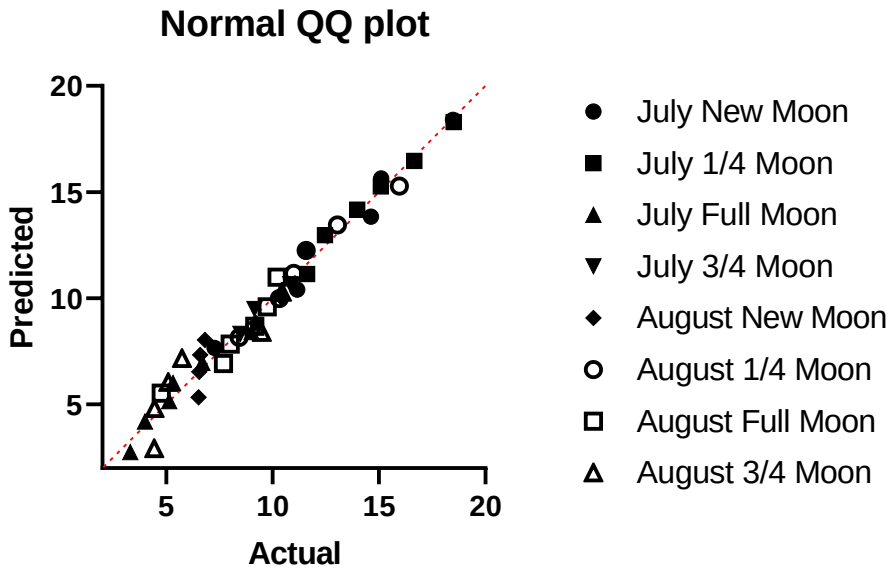
Supplements 3. D'Agostino and Pearson Omnibus normality test for catalase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

D'Agostino & Pearson normality test	
K2	1.532
P value	0.4649
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



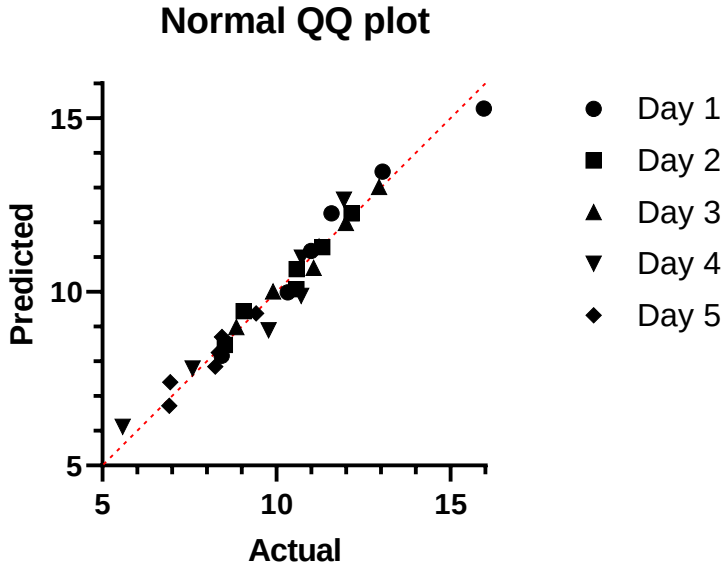
Supplements 4. D'Agostino and Pearson Omnibus normality test for glutathione reductase activity values over July and August reproductive timepoints.

D'Agostino & Pearson normality test	
K2	0.495
P value	0.7808
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



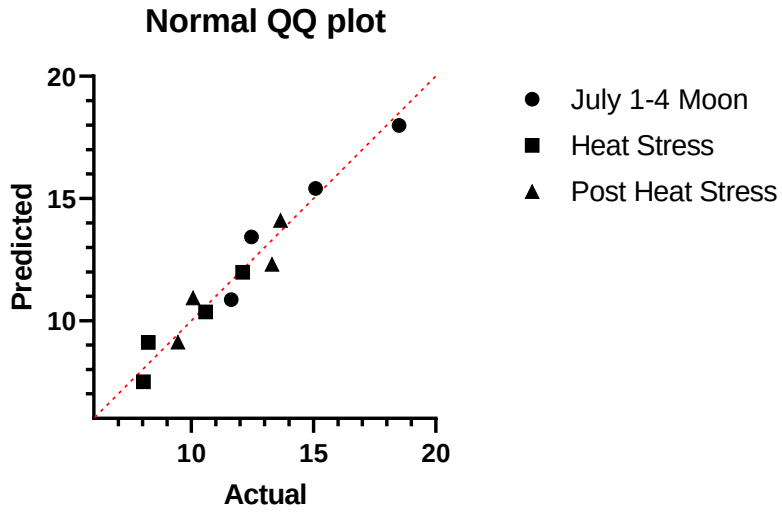
Supplements 5. D'Agostino and Pearson Omnibus normality test for glutathione reductase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

D'Agostino & Pearson normality test	
K2	1.535
P value	0.4642
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



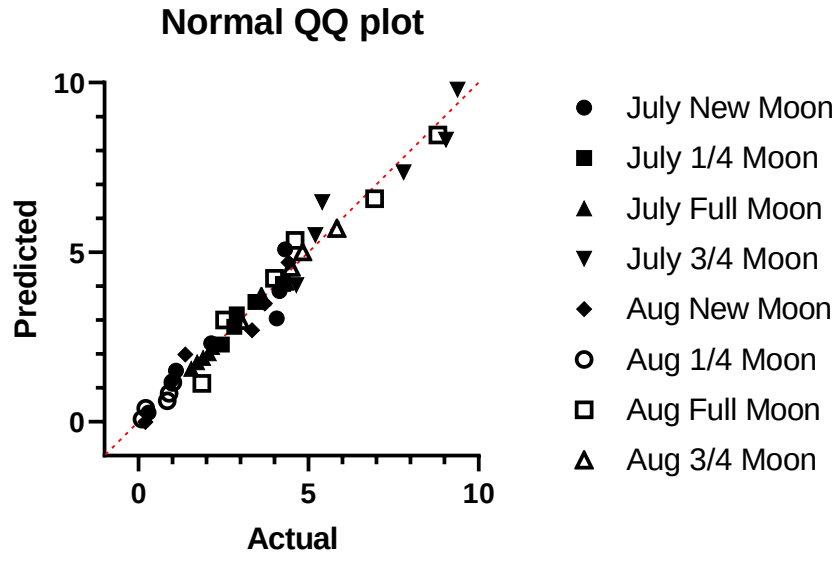
Supplements 6. D'Agostino and Pearson Omnibus normality test for glutathione reductase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

D'Agostino & Pearson normality test	
K2	2.214
P value	0.3306
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



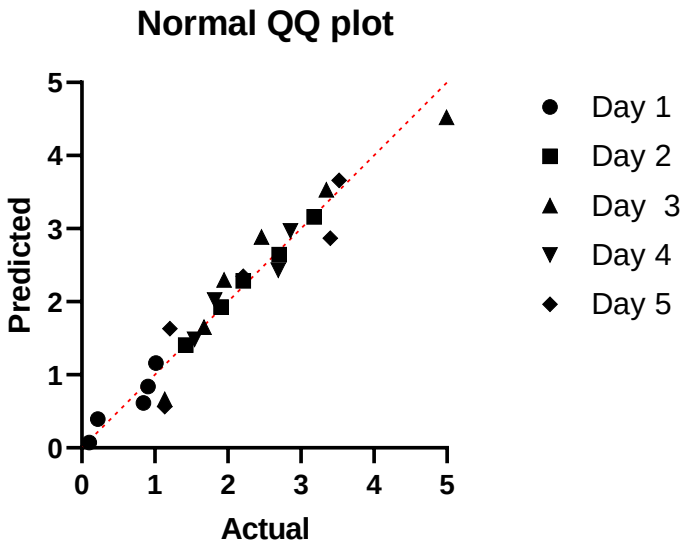
Supplements 7. D'Agostino and Pearson Omnibus normality test for Se-independent glutathione peroxidase activity values over July and August reproductive timepoints.

D'Agostino & Pearson normality test	
K2	5.963
P value	0.0507
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



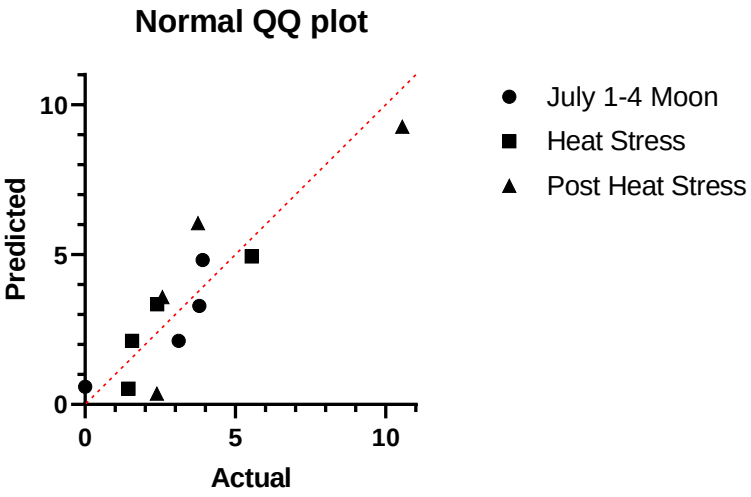
Supplements 8. D'Agostino and Pearson Omnibus normality test for Se-independent glutathione peroxidase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

D'Agostino & Pearson normality test	
K2	2.499
P value	0.2866
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



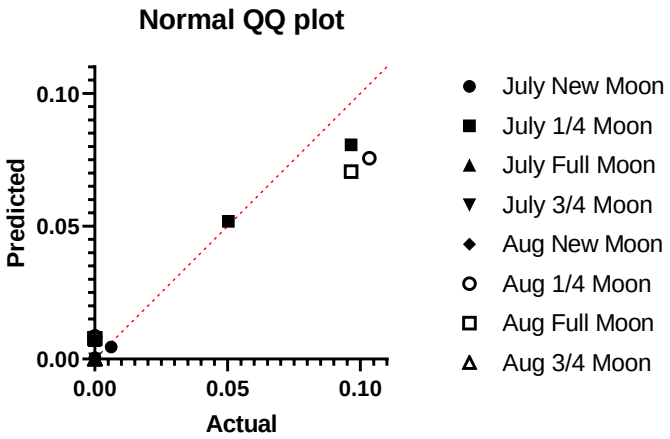
Supplements 9. D'Agostino and Pearson Omnibus normality test for Se-independent glutathione peroxidase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

D'Agostino & Pearson normality test	
K2	0.3531
P value	0.8382
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



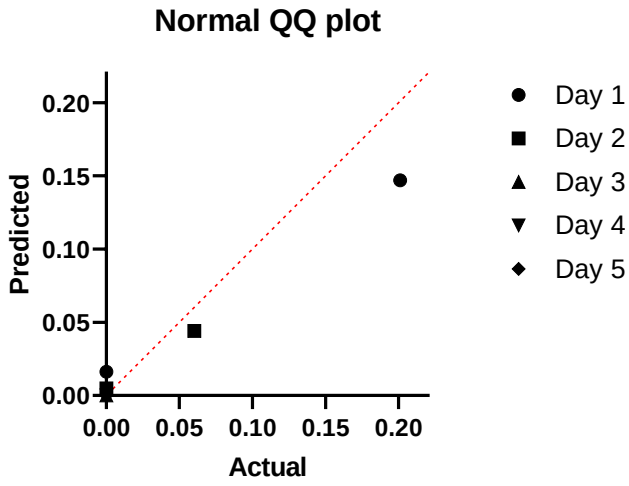
Supplements 10. D'Agostino and Pearson Omnibus normality test for Se-dependent glutathione peroxidase activity values over July and August reproductive timepoints. ***Note: Due to negligible values for enzyme activity in Se-dependent glutathione peroxidase assays, normality tests failed. This was expected, as data for enzyme activity for Se-dependent glutathione peroxidase suggests that this enzyme has little to no application for ROS detoxification in the S9 post-mitochondrial fraction of P. damicornis.***

D'Agostino & Pearson normality test	
K2	56.11
P value	<0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****



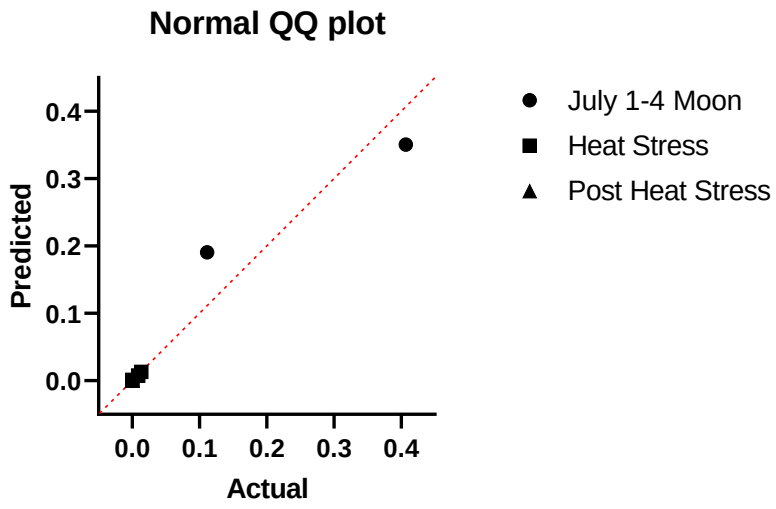
Supplements 11. D'Agostino and Pearson Omnibus normality test for Se-dependent glutathione peroxidase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak. ***Note: Due to negligible values for enzyme activity in Se-dependent glutathione peroxidase assays, normality tests failed. This was expected, as data for enzyme activity for Se-dependent glutathione peroxidase suggests that this enzyme has little to no application for ROS detoxification in the S9 post-mitochondrial fraction of P. damicornis.***

D'Agostino & Pearson normality test	
K2	65.74
P value	<0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****



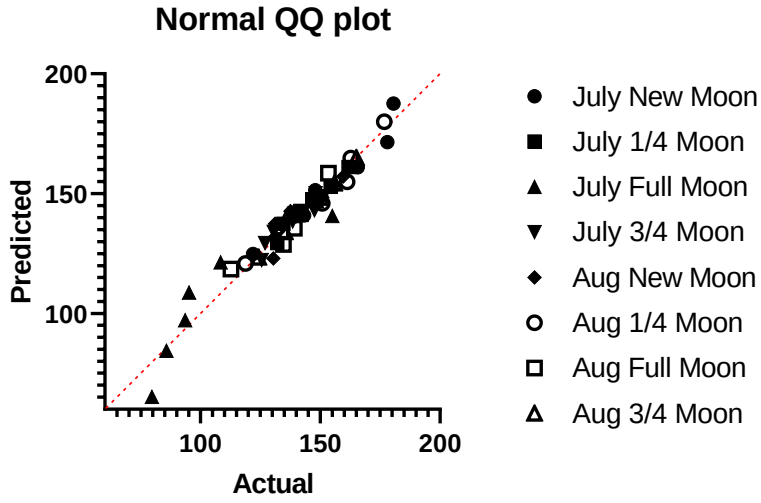
Supplements 12. D'Agostino and Pearson Omnibus normality test for Se-dependent glutathione peroxidase activity values over pre-bleaching, bleaching, and post-bleaching timepoints. ***Note: Due to negligible values for enzyme activity in Se-dependent glutathione peroxidase assays, normality tests failed. This was expected, as data for enzyme activity for Se-dependent glutathione peroxidase suggests that this enzyme has little to no application for ROS detoxification in the S9 post-mitochondrial fraction of P. damicornis.***

D'Agostino & Pearson normality test	
K2	28.77
P value	<0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****



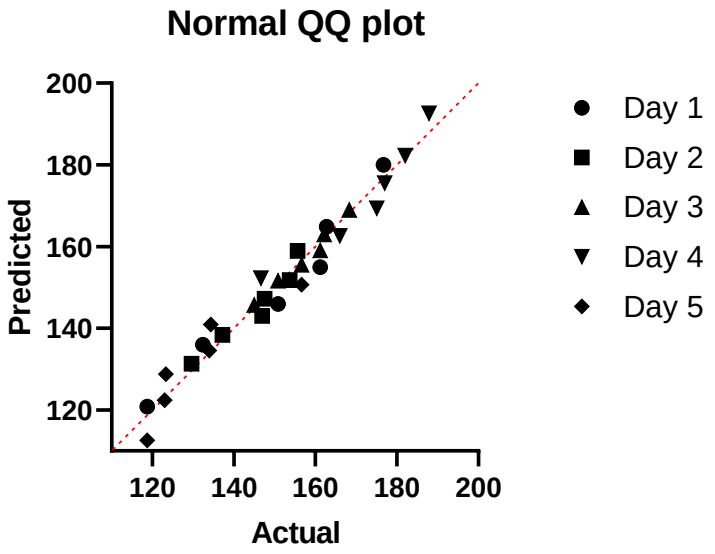
Supplements 13. D'Agostino and Pearson Omnibus normality test for superoxide dismutase activity values over July and August reproductive timepoints.

D'Agostino & Pearson normality test	
K2	4.886
P value	0.0869
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



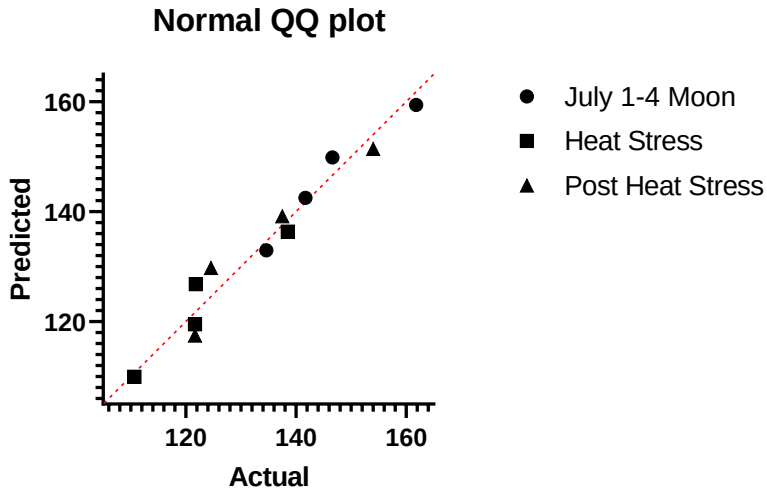
Supplements 14. D'Agostino and Pearson Omnibus normality test for superoxide dismutase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

D'Agostino & Pearson normality test	
K2	1.177
P value	0.5552
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



Supplements 15. D'Agostino and Pearson Omnibus normality test for superoxide dismutase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

D'Agostino & Pearson normality test	
K2	0.3289
P value	0.8484
Passed normality test (alpha=0.05)?	Yes
P value summary	ns



Supplements 16. One-way ANOVA test and Tukey's multiple comparisons test for catalase activity values over July and August reproductive timepoints.

ANOVA summary					
F	2.844				
P value	0.0177				
P value summary	*				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.3498				
Brown-Forsythe test					
F (DFn, DFd)	0.7523(7,37)				
P value	0.6299				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	46842758	7	6691823	F(7,37)=2.844	P=0.0177
Residual (within columns)	87056047	37	2352866		
Total	133898806	44			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	45				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July New Moon vs. July 1/4 Moon	1008	-2474 to 4490	No	ns	0.9811
July New Moon vs. July Full Moon	2330	-513.5 to 5173	No	ns	0.1775
July New Moon vs. July 3/4 Moon	793.6	-2049 to 3637	No	ns	0.9846
July New Moon vs. Aug New Moon	337.9	-2505 to 3181	No	ns	>0.9999
July New Moon vs. Aug 1/4 Moon	-1333	-4176 to 1510	No	ns	0.7996
July New Moon vs. Aug Full Moon	885.3	-1958 to 3728	No	ns	0.9716
July New Moon vs. Aug 3/4 Moon	1278	-1566 to 4121	No	ns	0.8315
July 1/4 Moon vs. July Full Moon	1322	-2160 to 4804	No	ns	0.9211
July 1/4 Moon vs. July 3/4 Moon	-214.1	-3696 to 3268	No	ns	>0.9999
July 1/4 Moon vs. Aug New Moon	-669.7	-4152 to 2812	No	ns	0.9984
July 1/4 Moon vs. Aug 1/4 Moon	-2341	-5823 to 1141	No	ns	0.3989
July 1/4 Moon vs. Aug Full Moon	-122.3	-3604 to 3360	No	ns	>0.9999
July 1/4 Moon vs. Aug 3/4 Moon	269.9	-3212 to 3752	No	ns	>0.9999
July Full Moon vs. July 3/4 Moon	-1536	-4379 to 1307	No	ns	0.6657
July Full Moon vs. Aug New Moon	-1992	-4835 to 851.5	No	ns	0.3478
July Full Moon vs. Aug 1/4 Moon	-3663	-6506 to -819.8	Yes	**	0.0044
July Full Moon vs. Aug Full Moon	-1444	-4287 to 1399	No	ns	0.7292
July Full Moon vs. Aug 3/4 Moon	-1052	-3895 to 1791	No	ns	0.9304
July 3/4 Moon vs. Aug New Moon	-455.7	-3299 to 2387	No	ns	0.9995
July 3/4 Moon vs. Aug 1/4 Moon	-2127	-4970 to 716.1	No	ns	0.2704

July 3/4 Moon vs. Aug Full Moon	91.74	-2751 to 2935	No	ns	>0.9999
July 3/4 Moon vs. Aug 3/4 Moon	483.9	-2359 to 3327	No	ns	0.9993
Aug New Moon vs. Aug 1/4 Moon	-1671	-4514 to 1172	No	ns	0.5678
Aug New Moon vs. Aug Full Moon	547.4	-2296 to 3390	No	ns	0.9984
Aug New Moon vs. Aug 3/4 Moon	939.6	-1903 to 3783	No	ns	0.9609
Aug 1/4 Moon vs. Aug Full Moon	2219	-624.4 to 5062	No	ns	0.2249
Aug 1/4 Moon vs. Aug 3/4 Moon	2611	-232.2 to 5454	No	ns	0.0915
Aug Full Moon vs. Aug 3/4 Moon	392.2	-2451 to 3235	No	ns	0.9998

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July New Moon vs. July 1/4 Moon	5919	4911	1008	1085	
July New Moon vs. July Full Moon	5919	3589	2330	885.6	
July New Moon vs. July 3/4 Moon	5919	5125	793.6	885.6	
July New Moon vs. Aug New Moon	5919	5581	337.9	885.6	
July New Moon vs. Aug 1/4 Moon	5919	7252	-1333	885.6	
July New Moon vs. Aug Full Moon	5919	5034	885.3	885.6	
July New Moon vs. Aug 3/4 Moon	5919	4641	1278	885.6	
July 1/4 Moon vs. July Full Moon	4911	3589	1322	1085	
July 1/4 Moon vs. July 3/4 Moon	4911	5125	-214.1	1085	
July 1/4 Moon vs. Aug New Moon	4911	5581	-669.7	1085	
July 1/4 Moon vs. Aug 1/4 Moon	4911	7252	-2341	1085	
July 1/4 Moon vs. Aug Full Moon	4911	5034	-122.3	1085	
July 1/4 Moon vs. Aug 3/4 Moon	4911	4641	269.9	1085	
July Full Moon vs. July 3/4 Moon	3589	5125	-1536	885.6	
July Full Moon vs. Aug New Moon	3589	5581	-1992	885.6	
July Full Moon vs. Aug 1/4 Moon	3589	7252	-3663	885.6	
July Full Moon vs. Aug Full Moon	3589	5034	-1444	885.6	
July Full Moon vs. Aug 3/4 Moon	3589	4641	-1052	885.6	
July 3/4 Moon vs. Aug New Moon	5125	5581	-455.7	885.6	
July 3/4 Moon vs. Aug 1/4 Moon	5125	7252	-2127	885.6	
July 3/4 Moon vs. Aug Full Moon	5125	5034	91.74	885.6	
July 3/4 Moon vs. Aug 3/4 Moon	5125	4641	483.9	885.6	
Aug New Moon vs. Aug 1/4 Moon	5581	7252	-1671	885.6	
Aug New Moon vs. Aug Full Moon	5581	5034	547.4	885.6	
Aug New Moon vs. Aug 3/4 Moon	5581	4641	939.6	885.6	
Aug 1/4 Moon vs. Aug Full Moon	7252	5034	2219	885.6	
Aug 1/4 Moon vs. Aug 3/4 Moon	7252	4641	2611	885.6	
Aug Full Moon vs. Aug 3/4 Moon	5034	4641	392.2	885.6	

Supplements 17. One-way ANOVA test and Tukey's multiple comparisons test for catalase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

ANOVA summary					
F	1.444				
P value	0.2374				
P value summary	ns				
Sig. diff. among means (P < 0.05)?	No				
R square	0.194				
Brown-Forsythe test					
F (DFn, DFd)	1.488(5,30)				
P value	0.2233				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	9.521				
P value	0.0900				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	29403748	5	5880750	F(5,30)=1.444	P=0.2374
Residual (within columns)	122159800	30	4071993		
Total	151563548	35			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	36				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
day 1 vs. day 2	1069	-2475 to 4612	No	ns	0.9389
day 1 vs. day 3	813.6	-2730 to 4357	No	ns	0.9807
day 1 vs. day 4	-483.2	-4027 to 3060	No	ns	0.9983
day 1 vs. day 5	1542	-2002 to 5086	No	ns	0.7699
day 1 vs. Aug Full Moon	2219	-1325 to 5762	No	ns	0.4192
day 2 vs. day 3	-255.2	-3799 to 3288	No	ns	>0.9999
day 2 vs. day 4	-1552	-5096 to 1992	No	ns	0.7652
day 2 vs. day 5	473.2	-3070 to 4017	No	ns	0.9984
day 2 vs. Aug Full Moon	1150	-2394 to 4693	No	ns	0.9185
day 3 vs. day 4	-1297	-4840 to 2247	No	ns	0.8721
day 3 vs. day 5	728.5	-2815 to 4272	No	ns	0.9882
day 3 vs. Aug Full Moon	1405	-2139 to 4949	No	ns	0.8305
day 4 vs. day 5	2025	-1518 to 5569	No	ns	0.5186
day 4 vs. Aug Full Moon	2702	-841.8 to 6245	No	ns	0.2180
day 5 vs. Aug Full Moon	676.6	-2867 to 4220	No	ns	0.9916
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
day 1 vs. day 2	7252	6183	1069	1165	
day 1 vs. day 3	7252	6439	813.6	1165	
day 1 vs. day 4	7252	7735	-483.2	1165	
day 1 vs. day 5	7252	5710	1542	1165	
day 1 vs. Aug Full Moon	7252	5034	2219	1165	
day 2 vs. day 3	6183	6439	-255.2	1165	

day 2 vs. day 4	6183	7735	-1552	1165	
day 2 vs. day 5	6183	5710	473.2	1165	
day 2 vs. Aug Full Moon	6183	5034	1150	1165	
day 3 vs. day 4	6439	7735	-1297	1165	
day 3 vs. day 5	6439	5710	728.5	1165	
day 3 vs. Aug Full Moon	6439	5034	1405	1165	
day 4 vs. day 5	7735	5710	2025	1165	
day 4 vs. Aug Full Moon	7735	5034	2702	1165	
day 5 vs. Aug Full Moon	5710	5034	676.6	1165	

Supplements 18. One-way ANOVA test and Tukey's multiple comparisons test for catalase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

ANOVA summary					
F	20.08				
P value	0.0005				
P value summary	***				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.817				
Brown-Forsythe test					
F (DFn, DFd)	1.289(2,9)				
P value	0.3220				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	5.453				
P value	0.0655				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	8824457	2	4412229	F(2,9)=20.08	P=0.0005
Residual (within columns)	1977122	9	219680		
Total	10801579	11			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	12				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July 1-4 Moon vs. Heat Stress	-1578	-2503 to -652.7	Yes	**	0.0026
July 1-4 Moon vs. Post Heat Stress	411.7	-513.6 to 1337	No	ns	0.4598
Heat Stress vs. Post Heat Stress	1990	1064 to 2915	Yes	***	0.0005
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July 1-4 Moon vs. Heat Stress	3431	5009	-1578	331.4	
July 1-4 Moon vs. Post Heat Stress	3431	3019	411.7	331.4	
Heat Stress vs. Post Heat Stress	5009	3019	1990	331.4	

Supplements 19. One-way ANOVA test and Tukey's multiple comparisons test for glutathione reductase activity values over July and August reproductive timepoints.

ANOVA summary					
F	9.846				
P value	<0.0001				
P value summary	****				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.6507				
Brown-Forsythe test					
F (DFn, DFd)	0.9389(7,37)				
P value	0.4888				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	437.8	7	62.54	F(7,37)=9.846	P<0.0001
Residual (within columns)	235.0	37	6.352		
Total	672.8	44			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	45				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July New Moon vs. July 1/4 Moon	-1.690	-6.362 to 2.981	No	ns	0.9377
July New Moon vs. July Full Moon	7.446	2.774 to 12.12	Yes	***	0.0002
July New Moon vs. July 3/4 Moon	3.539	-2.182 to 9.260	No	ns	0.5045
July New Moon vs. August New Moon	5.342	0.6710 to 10.01	Yes	*	0.0156
July New Moon vs. August 1/4 Moon	1.311	-3.361 to 5.982	No	ns	0.9841
July New Moon vs. August Full Moon	4.766	0.09437 to 9.437	Yes	*	0.0428
July New Moon vs. August 3/4 Moon	6.420	1.748 to 11.09	Yes	**	0.0020
July 1/4 Moon vs. July Full Moon	9.136	4.465 to 13.81	Yes	****	<0.0001
July 1/4 Moon vs. July 3/4 Moon	5.230	-0.4916 to 10.95	No	ns	0.0943
July 1/4 Moon vs. August New Moon	7.033	2.361 to 11.70	Yes	***	0.0006
July 1/4 Moon vs. August 1/4 Moon	3.001	-1.670 to 7.672	No	ns	0.4565
July 1/4 Moon vs. August Full Moon	6.456	1.785 to 11.13	Yes	**	0.0018
July 1/4 Moon vs. August 3/4 Moon	8.110	3.439 to 12.78	Yes	****	<0.0001
July Full Moon vs. July 3/4 Moon	-3.907	-9.628 to 1.815	No	ns	0.3795
July Full Moon vs. August New Moon	-2.103	-6.775 to 2.568	No	ns	0.8300

July Full Moon vs. August 1/4 Moon	-6.135	-10.81 to -1.464	Yes	**	0.0035
July Full Moon vs. August Full Moon	-2.680	-7.351 to 1.991	No	ns	0.5971
July Full Moon vs. August 3/4 Moon	-1.026	-5.697 to 3.645	No	ns	0.9963
July 3/4 Moon vs. August New Moon	1.803	-3.918 to 7.524	No	ns	0.9697
July 3/4 Moon vs. August 1/4 Moon	-2.228	-7.950 to 3.493	No	ns	0.9108
July 3/4 Moon vs. August Full Moon	1.227	-4.495 to 6.948	No	ns	0.9968
July 3/4 Moon vs. August 3/4 Moon	2.880	-2.841 to 8.602	No	ns	0.7377
August New Moon vs. August 1/4 Moon	-4.032	-8.703 to 0.6397	No	ns	0.1339
August New Moon vs. August Full Moon	-0.5767	-5.248 to 4.095	No	ns	>0.9999
August New Moon vs. August 3/4 Moon	1.077	-3.594 to 5.749	No	ns	0.9950
August 1/4 Moon vs. August Full Moon	3.455	-1.216 to 8.126	No	ns	0.2834
August 1/4 Moon vs. August 3/4 Moon	5.109	0.4375 to 9.780	Yes	*	0.0237
August Full Moon vs. August 3/4 Moon	1.654	-3.017 to 6.325	No	ns	0.9441
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1
July New Moon vs. July 1/4 Moon	13.03	14.72	-1.690	1.455	6
July New Moon vs. July Full Moon	13.03	5.583	7.446	1.455	6
July New Moon vs. July 3/4 Moon	13.03	9.489	3.539	1.782	6
July New Moon vs. August New Moon	13.03	7.686	5.342	1.455	6
July New Moon vs. August 1/4 Moon	13.03	11.72	1.311	1.455	6
July New Moon vs. August Full Moon	13.03	8.263	4.766	1.455	6
July New Moon vs. August 3/4 Moon	13.03	6.609	6.420	1.455	6
July 1/4 Moon vs. July Full Moon	14.72	5.583	9.136	1.455	6
July 1/4 Moon vs. July 3/4 Moon	14.72	9.489	5.230	1.782	6
July 1/4 Moon vs. August New Moon	14.72	7.686	7.033	1.455	6
July 1/4 Moon vs. August 1/4 Moon	14.72	11.72	3.001	1.455	6
July 1/4 Moon vs. August Full Moon	14.72	8.263	6.456	1.455	6
July 1/4 Moon vs. August 3/4 Moon	14.72	6.609	8.110	1.455	6
July Full Moon vs. July 3/4 Moon	5.583	9.489	-3.907	1.782	6
July Full Moon vs. August New Moon	5.583	7.686	-2.103	1.455	6
July Full Moon vs. August 1/4 Moon	5.583	11.72	-6.135	1.455	6
July Full Moon vs. August Full Moon	5.583	8.263	-2.680	1.455	6
July Full Moon vs. August 3/4 Moon	5.583	6.609	-1.026	1.455	6
July 3/4 Moon vs. August New Moon	9.489	7.686	1.803	1.782	3
July 3/4 Moon vs. August 1/4 Moon	9.489	11.72	-2.228	1.782	3
July 3/4 Moon vs. August Full Moon	9.489	8.263	1.227	1.782	3
July 3/4 Moon vs. August 3/4 Moon	9.489	6.609	2.880	1.782	3
August New Moon vs. August 1/4 Moon	7.686	11.72	-4.032	1.455	6

August New Moon vs. August Full Moon	7.686	8.263	-0.5767	1.455	6
August New Moon vs. August 3/4 Moon	7.686	6.609	1.077	1.455	6
August 1/4 Moon vs. August Full Moon	11.72	8.263	3.455	1.455	6
August 1/4 Moon vs. August 3/4 Moon	11.72	6.609	5.109	1.455	6
August Full Moon vs. August 3/4 Moon	8.263	6.609	1.654	1.455	6

Supplements 20. One-way ANOVA test and Tukey's multiple comparisons test for glutathione reductase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

ANOVA summary					
F	3.598				
P value	0.0189				
P value summary	*				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.3653				
Brown-Forsythe test					
F (DFn, DFd)	0.9472(4,25)				
P value	0.4533				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	5.759				
P value	0.2179				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	49.29	4	12.32	F(4,25)=3.598	P=0.0189
Residual (within columns)	85.63	25	3.425		
Total	134.9	29			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	30				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
day 1 vs. day 2	1.355	-1.784 to 4.493	No	ns	0.7126
day 1 vs. day 3	0.7215	-2.417 to 3.860	No	ns	0.9600
day 1 vs. day 4	2.333	-0.8053 to 5.471	No	ns	0.2187
day 1 vs. day 5	3.671	0.5326 to 6.809	Yes	*	0.0162
day 2 vs. day 3	-0.6331	-3.771 to 2.505	No	ns	0.9750
day 2 vs. day 4	0.9782	-2.160 to 4.116	No	ns	0.8883
day 2 vs. day 5	2.316	-0.8220 to 5.454	No	ns	0.2245
day 3 vs. day 4	1.611	-1.527 to 4.749	No	ns	0.5670
day 3 vs. day 5	2.949	-0.1889 to 6.087	No	ns	0.0728
day 4 vs. day 5	1.338	-1.800 to 4.476	No	ns	0.7217

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
day 1 vs. day 2	11.72	10.36	1.355	1.069	
day 1 vs. day 3	11.72	11.00	0.7215	1.069	
day 1 vs. day 4	11.72	9.385	2.333	1.069	
day 1 vs. day 5	11.72	8.047	3.671	1.069	
day 2 vs. day 3	10.36	11.00	-0.6331	1.069	
day 2 vs. day 4	10.36	9.385	0.9782	1.069	
day 2 vs. day 5	10.36	8.047	2.316	1.069	
day 3 vs. day 4	11.00	9.385	1.611	1.069	
day 3 vs. day 5	11.00	8.047	2.949	1.069	
day 4 vs. day 5	9.385	8.047	1.338	1.069	

Supplements 21. One-way ANOVA test and Tukey's multiple comparisons test for glutathione reductase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

ANOVA summary					
F	3.687				
P value	0.0677				
P value summary	ns				
Sig. diff. among means (P < 0.05)?	No				
R square	0.4503				
Brown-Forsythe test					
F (DFn, DFd)	0.5818(2, 9)				
P value	0.5786				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	0.6489				
P value	0.7229				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	44.44	2	22.22	F(2,9)=3.687	P=0.0677
Residual (within columns)	54.25	9	6.027		
Total	98.69	11			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	12				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July 1-4 Moon vs. Heat Stress	4.684	-0.1633 to 9.531	No	ns	0.0579
July 1-4 Moon vs. Post Heat Stress	2.803	-2.044 to 7.65	No	ns	0.2888
Heat Stress vs. Post Heat Stress	-1.88	-6.727 to 2.967	No	ns	0.5472
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1
July 1-4 Moon vs. Heat Stress	14.42	9.735	4.684	1.736	4
July 1-4 Moon vs. Post Heat Stress	14.42	11.62	2.803	1.736	4
Heat Stress vs. Post Heat Stress	9.735	11.62	-1.88	1.736	4

Supplements 22. One-way ANOVA test and Tukey's multiple comparisons test for Se-independent glutathione peroxidase activity values over July and August reproductive timepoints.

ANOVA summary					
F	8.352				
P value	<0.0001				
P value summary	****				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.6061				
Brown-Forsythe test					
F (DFn, DFd)	4.058(7,38)				
P value	0.0021				
P value summary	**				
SDs significantly different (P < 0.05)?	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	18.94				
P value	0.0084				
P value summary	**				
SDs significantly different (P < 0.05)?	Yes				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	148.7	7	21.25	F(7,38)=8.352	P<0.0001
Residual (within columns)	96.67	38	2.544		
Total	245.4	45			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	46				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July New Moon vs. July 1/4 Moon	-0.4907	-3.587 to 2.605	No	ns	0.9996
July New Moon vs. July Full Moon	0.5044	-2.447 to 3.456	No	ns	0.9993
July New Moon vs. July 3/4 Moon	-4.234	-7.186 to -1.282	Yes	**	0.0011
July New Moon vs. Aug New Moon	0.334	-2.618 to 3.286	No	ns	>0.9999
July New Moon vs. Aug 1/4 Moon	2.062	-1.034 to 5.158	No	ns	0.4122
July New Moon vs. Aug Full Moon	-2.114	-5.065 to 0.838	No	ns	0.3224
July New Moon vs. Aug 3/4 Moon	-1.664	-4.616 to 1.287	No	ns	0.6192
July 1/4 Moon vs. July Full Moon	0.9951	-2.101 to 4.091	No	ns	0.9667
July 1/4 Moon vs. July 3/4 Moon	-3.743	-6.839 to -0.6475	Yes	**	0.0088
July 1/4 Moon vs. Aug New Moon	0.8247	-2.271 to 3.921	No	ns	0.9884
July 1/4 Moon vs. Aug 1/4 Moon	2.553	-0.6809 to 5.786	No	ns	0.2139
July 1/4 Moon vs. Aug Full Moon	-1.623	-4.719 to 1.473	No	ns	0.6992
July 1/4 Moon vs. Aug 3/4 Moon	-1.174	-4.269 to 1.922	No	ns	0.9223
July Full Moon vs. July 3/4 Moon	-4.738	-7.69 to -1.787	Yes	***	0.0002
July Full Moon vs. Aug New Moon	-0.1703	-3.122 to 2.781	No	ns	>0.9999
July Full Moon vs. Aug 1/4 Moon	1.558	-1.538 to 4.653	No	ns	0.7399
July Full Moon vs. Aug Full Moon	-2.618	-5.57 to 0.3336	No	ns	0.1142
July Full Moon vs. Aug 3/4 Moon	-2.169	-5.12 to 0.7831	No	ns	0.2921
July 3/4 Moon vs. Aug New Moon	4.568	1.616 to 7.52	Yes	***	0.0004
July 3/4 Moon vs. Aug 1/4 Moon	6.296	3.2 to 9.392	Yes	****	<0.0001
July 3/4 Moon vs. Aug Full Moon	2.12	-0.8314 to	No	ns	0.3186

		5.072			
July 3/4 Moon vs. Aug 3/4 Moon	2.57	-0.382 to 5.521	No	ns	0.1277
Aug New Moon vs. Aug 1/4 Moon	1.728	-1.368 to 4.824	No	ns	0.6309
Aug New Moon vs. Aug Full Moon	-2.448	-5.4 to 0.504	No	ns	0.1673
Aug New Moon vs. Aug 3/4 Moon	-1.998	-4.95 to 0.9534	No	ns	0.3915
Aug 1/4 Moon vs. Aug Full Moon	-4.176	-7.271 to -1.08	Yes	**	0.0025
Aug 1/4 Moon vs. Aug 3/4 Moon	-3.726	-6.822 to -0.6304	Yes	**	0.0092
Aug Full Moon vs. Aug 3/4 Moon	0.4494	-2.502 to 3.401	No	ns	0.9997
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July New Moon vs. July 1/4 Moon	2.678	3.168	-0.4907	0.9658	
July New Moon vs. July Full Moon	2.678	2.173	0.5044	0.9209	
July New Moon vs. July 3/4 Moon	2.678	6.912	-4.234	0.9209	
July New Moon vs. Aug New Moon	2.678	2.344	0.334	0.9209	
July New Moon vs. Aug 1/4 Moon	2.678	0.6158	2.062	0.9658	
July New Moon vs. Aug Full Moon	2.678	4.791	-2.114	0.9209	
July New Moon vs. Aug 3/4 Moon	2.678	4.342	-1.664	0.9209	
July 1/4 Moon vs. July Full Moon	3.168	2.173	0.9951	0.9658	
July 1/4 Moon vs. July 3/4 Moon	3.168	6.912	-3.743	0.9658	
July 1/4 Moon vs. Aug New Moon	3.168	2.344	0.8247	0.9658	
July 1/4 Moon vs. Aug 1/4 Moon	3.168	0.6158	2.553	1.009	
July 1/4 Moon vs. Aug Full Moon	3.168	4.791	-1.623	0.9658	
July 1/4 Moon vs. Aug 3/4 Moon	3.168	4.342	-1.174	0.9658	
July Full Moon vs. July 3/4 Moon	2.173	6.912	-4.738	0.9209	
July Full Moon vs. Aug New Moon	2.173	2.344	-0.1703	0.9209	
July Full Moon vs. Aug 1/4 Moon	2.173	0.6158	1.558	0.9658	
July Full Moon vs. Aug Full Moon	2.173	4.791	-2.618	0.9209	
July Full Moon vs. Aug 3/4 Moon	2.173	4.342	-2.169	0.9209	
July 3/4 Moon vs. Aug New Moon	6.912	2.344	4.568	0.9209	
July 3/4 Moon vs. Aug 1/4 Moon	6.912	0.6158	6.296	0.9658	
July 3/4 Moon vs. Aug Full Moon	6.912	4.791	2.12	0.9209	
July 3/4 Moon vs. Aug 3/4 Moon	6.912	4.342	2.57	0.9209	
Aug New Moon vs. Aug 1/4 Moon	2.344	0.6158	1.728	0.9658	
Aug New Moon vs. Aug Full Moon	2.344	4.791	-2.448	0.9209	
Aug New Moon vs. Aug 3/4 Moon	2.344	4.342	-1.998	0.9209	
Aug 1/4 Moon vs. Aug Full Moon	0.6158	4.791	-4.176	0.9658	
Aug 1/4 Moon vs. Aug 3/4 Moon	0.6158	4.342	-3.726	0.9658	
Aug Full Moon vs. Aug 3/4 Moon	4.791	4.342	0.4494	0.9209	

Supplements 23. One-way ANOVA test and Tukey's multiple comparisons test for Se-independent glutathione peroxidase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

ANOVA summary					
F	3.276				
P value	0.0310				
P value summary	*				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.3842				
Brown-Forsythe test					
F (DFn, DFd)	1.282(4,21)				
P value	0.3087				

P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	6.265				
P value	0.1802				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	12.37	4	3.092	F(4,21)=3.276	P=0.0310
Residual (within columns)	19.82	21	0.9440		
Total	32.19	25			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	26				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
day 1 vs. day 2	-1.669	-3.499 to 0.1617	No	ns	0.0851
day 1 vs. day 3	-1.976	-3.729 to -0.2237	Yes	*	0.0222
day 1 vs. day 4	-1.612	-3.553 to 0.3297	No	ns	0.1351
day 1 vs. day 5	-1.497	-3.250 to 0.2556	No	ns	0.1183
day 2 vs. day 3	-0.3075	-2.060 to 1.445	No	ns	0.9840
day 2 vs. day 4	0.05691	-1.885 to 1.999	No	ns	>0.9999
day 2 vs. day 5	0.1718	-1.581 to 1.924	No	ns	0.9983
day 3 vs. day 4	0.3645	-1.504 to 2.233	No	ns	0.9764
day 3 vs. day 5	0.4793	-1.192 to 2.150	No	ns	0.9101
day 4 vs. day 5	0.1148	-1.753 to 1.983	No	ns	0.9997
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
day 1 vs. day 2	0.6158	2.285	-1.669	0.6145	
day 1 vs. day 3	0.6158	2.592	-1.976	0.5883	
day 1 vs. day 4	0.6158	2.228	-1.612	0.6518	
day 1 vs. day 5	0.6158	2.113	-1.497	0.5883	
day 2 vs. day 3	2.285	2.592	-0.3075	0.5883	
day 2 vs. day 4	2.285	2.228	0.05691	0.6518	
day 2 vs. day 5	2.285	2.113	0.1718	0.5883	
day 3 vs. day 4	2.592	2.228	0.3645	0.6271	
day 3 vs. day 5	2.592	2.113	0.4793	0.5609	
day 4 vs. day 5	2.228	2.113	0.1148	0.6271	

Supplements 24. One-way ANOVA test and Tukey's multiple comparisons test for Se-independent glutathione peroxidase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

ANOVA summary					
F	0.7967				
P value	0.4802				
P value summary	ns				
Sig. diff. among means (P < 0.05)?	No				
R square	0.1504				
Brown-Forsythe test					
F (DFn, DFd)	0.3264(2,9)				
P value	0.7297				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	1.978				
P value	0.3719				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	11.69	2	5.847	F(2,9)=0.7967	P=0.4802
Residual (within columns)	66.06	9	7.340		
Total	77.75	11			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	12				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July 1-4 Moon vs. Heat Stress	-0.03161	-5.380 to 5.317	No	ns	0.9998
July 1-4 Moon vs. Post Heat Stress	-2.110	-7.458 to 3.239	No	ns	0.5369
Heat Stress vs. Post Heat Stress	-2.078	-7.427 to 3.270	No	ns	0.5463
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July 1-4 Moon vs. Heat Stress	2.704	2.735	-0.03161	1.916	
July 1-4 Moon vs. Post Heat Stress	2.704	4.813	-2.110	1.916	
Heat Stress vs. Post Heat Stress	2.735	4.813	-2.078	1.916	

Supplements 25. One-way ANOVA test and Tukey's multiple comparisons test for Se-dependent glutathione peroxidase activity values over July and August reproductive timepoints.

ANOVA summary					
F	0.9932				
P value	0.4502				
P value summary	ns				
Sig. diff. among means (P < 0.05)?	No				
R square	0.1481				
Brown-Forsythe test					
F (DFn, DFd)	0.9932(7,40)				
P value	0.4502				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	+infinity				
P value	<0.0001				
P value summary	****				
SDs significantly different (P < 0.05)?	Yes				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	0.004333	7	0.000619	F(7,40)=0.9932	P=0.4502
Residual (within columns)	0.02493	40	0.0006232		
Total	0.02926	47			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	48				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July New Moon vs. July 1/4 Moon	-0.02342	-0.06949 to 0.02265	No	ns	0.7329
July New Moon vs. July Full Moon	0.001031	-0.04504 to 0.0471	No	ns	>0.9999
July New Moon vs. July 3/4 Moon	0.001031	-0.04504 to 0.0471	No	ns	>0.9999
July New Moon vs. Aug New Moon	0.001031	-0.04504 to 0.0471	No	ns	>0.9999
July New Moon vs. Aug 1/4 Moon	-0.01619	-0.06227 to 0.02988	No	ns	0.9477
July New Moon vs. Aug Full Moon	-0.01505	-0.06112 to 0.03103	No	ns	0.9644
July New Moon vs. Aug 3/4 Moon	0.001031	-0.04504 to 0.0471	No	ns	>0.9999
July 1/4 Moon vs. July Full Moon	0.02445	-0.02162 to 0.07052	No	ns	0.6894
July 1/4 Moon vs. July 3/4 Moon	0.02445	-0.02162 to 0.07052	No	ns	0.6894

July 1/4 Moon vs. Aug New Moon	0.02445	-0.02162 to 0.07052	No	ns	0.6894
July 1/4 Moon vs. Aug 1/4 Moon	0.007225	-0.03885 to 0.0533	No	ns	0.9996
July 1/4 Moon vs. Aug Full Moon	0.008374	-0.0377 to 0.05445	No	ns	0.9989
July 1/4 Moon vs. Aug 3/4 Moon	0.02445	-0.02162 to 0.07052	No	ns	0.6894
July Full Moon vs. July 3/4 Moon	0	-0.04607 to 0.04607	No	ns	>0.9999
July Full Moon vs. Aug New Moon	0	-0.04607 to 0.04607	No	ns	>0.9999
July Full Moon vs. Aug 1/4 Moon	-0.01723	-0.0633 to 0.02885	No	ns	0.9286
July Full Moon vs. Aug Full Moon	-0.01608	-0.06215 to 0.03	No	ns	0.9496
July Full Moon vs. Aug 3/4 Moon	0	-0.04607 to 0.04607	No	ns	>0.9999
July 3/4 Moon vs. Aug New Moon	0	-0.04607 to 0.04607	No	ns	>0.9999
July 3/4 Moon vs. Aug 1/4 Moon	-0.01723	-0.0633 to 0.02885	No	ns	0.9286
July 3/4 Moon vs. Aug Full Moon	-0.01608	-0.06215 to 0.03	No	ns	0.9496
July 3/4 Moon vs. Aug 3/4 Moon	0	-0.04607 to 0.04607	No	ns	>0.9999
Aug New Moon vs. Aug 1/4 Moon	-0.01723	-0.0633 to 0.02885	No	ns	0.9286
Aug New Moon vs. Aug Full Moon	-0.01608	-0.06215 to 0.03	No	ns	0.9496
Aug New Moon vs. Aug 3/4 Moon	0	-0.04607 to 0.04607	No	ns	>0.9999
Aug 1/4 Moon vs. Aug Full Moon	0.001149	-0.04492 to 0.04722	No	ns	>0.9999
Aug 1/4 Moon vs. Aug 3/4 Moon	0.01723	-0.02885 to 0.0633	No	ns	0.9286
Aug Full Moon vs. Aug 3/4 Moon	0.01608	-0.03 to 0.06215	No	ns	0.9496
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July New Moon vs. July 1/4 Moon	0.001031	0.02445	-0.02342	0.01441	

July New Moon vs. July Full Moon	0.001031	0	0.001031	0.01441	
July New Moon vs. July 3/4 Moon	0.001031	0	0.001031	0.01441	
July New Moon vs. Aug New Moon	0.001031	0	0.001031	0.01441	
July New Moon vs. Aug 1/4 Moon	0.001031	0.01723	-0.01619	0.01441	
July New Moon vs. Aug Full Moon	0.001031	0.01608	-0.01505	0.01441	
July New Moon vs. Aug 3/4 Moon	0.001031	0	0.001031	0.01441	
July 1/4 Moon vs. July Full Moon	0.02445	0	0.02445	0.01441	
July 1/4 Moon vs. July 3/4 Moon	0.02445	0	0.02445	0.01441	
July 1/4 Moon vs. Aug New Moon	0.02445	0	0.02445	0.01441	
July 1/4 Moon vs. Aug 1/4 Moon	0.02445	0.01723	0.007225	0.01441	
July 1/4 Moon vs. Aug Full Moon	0.02445	0.01608	0.008374	0.01441	
July 1/4 Moon vs. Aug 3/4 Moon	0.02445	0	0.02445	0.01441	
July Full Moon vs. July 3/4 Moon	0	0	0	0.01441	
July Full Moon vs. Aug New Moon	0	0	0	0.01441	
July Full Moon vs. Aug 1/4 Moon	0	0.01723	-0.01723	0.01441	
July Full Moon vs. Aug Full Moon	0	0.01608	-0.01608	0.01441	
July Full Moon vs. Aug 3/4 Moon	0	0	0	0.01441	
July 3/4 Moon vs. Aug New Moon	0	0	0	0.01441	
July 3/4 Moon vs. Aug 1/4 Moon	0	0.01723	-0.01723	0.01441	
July 3/4 Moon vs. Aug Full Moon	0	0.01608	-0.01608	0.01441	
July 3/4 Moon vs. Aug 3/4 Moon	0	0	0	0.01441	
Aug New Moon vs. Aug 1/4 Moon	0	0.01723	-0.01723	0.01441	
Aug New Moon vs. Aug Full Moon	0	0.01608	-0.01608	0.01441	
Aug New Moon vs. Aug 3/4 Moon	0	0	0	0.01441	
Aug 1/4 Moon vs. Aug Full Moon	0.01723	0.01608	0.001149	0.01441	
Aug 1/4 Moon vs. Aug 3/4 Moon	0.01723	0	0.01723	0.01441	
Aug Full Moon vs. Aug 3/4 Moon	0.01608	0	0.01608	0.01441	

Supplements 26. One-way ANOVA test and Tukey's multiple comparisons test for Se-dependent glutathione peroxidase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

ANOVA summary					
F	0.8624				
P value	0.5000				
P value summary	ns				
Sig. diff. among means (P < 0.05)?	No				
R square	0.1213				
Brown-Forsythe test					
F (DFn, DFd)	0.8624(4,25)				
P value	0.5000				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	+infinity				
P value	<0.0001				
P value summary	****				
SDs significantly different (P < 0.05)?	Yes				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	0.005062	4	0.001265	F(4,25) =0.8624	P=0.5000

Residual (within columns)	0.03668	25	0.001467		
Total	0.04174	29			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	30				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
day 1 vs. day 2	0.02345	-0.04151 to 0.0884	No	ns	0.8248
day 1 vs. day 3	0.03349	-0.03146 to 0.09844	No	ns	0.5631
day 1 vs. day 4	0.03349	-0.03146 to 0.09844	No	ns	0.5631
day 1 vs. day 5	0.03349	-0.03146 to 0.09844	No	ns	0.5631
day 2 vs. day 3	0.01005	-0.0549 to 0.075	No	ns	0.9907
day 2 vs. day 4	0.01005	-0.0549 to 0.075	No	ns	0.9907
day 2 vs. day 5	0.01005	-0.0549 to 0.075	No	ns	0.9907
day 3 vs. day 4	0	-0.06495 to 0.06495	No	ns	>0.9999
day 3 vs. day 5	0	-0.06495 to 0.06495	No	ns	>0.9999
day 4 vs. day 5	0	-0.06495 to 0.06495	No	ns	>0.9999
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
day 1 vs. day 2	0.03349	0.01005	0.02345	0.02212	
day 1 vs. day 3	0.03349	0	0.03349	0.02212	
day 1 vs. day 4	0.03349	0	0.03349	0.02212	
day 1 vs. day 5	0.03349	0	0.03349	0.02212	
day 2 vs. day 3	0.01005	0	0.01005	0.02212	
day 2 vs. day 4	0.01005	0	0.01005	0.02212	
day 2 vs. day 5	0.01005	0	0.01005	0.02212	
day 3 vs. day 4	0	0	0	0.02212	
day 3 vs. day 5	0	0	0	0.02212	
day 4 vs. day 5	0	0	0	0.02212	

Supplements 27. One-way ANOVA test and Tukey's multiple comparisons test for Se-dependent glutathione peroxidase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

P value summary	ns				
Sig. diff. among means (P < 0.05)?	No				
R square	0.2789				
Brown-Forsythe test					
F (DFn, DFd)	2.945(2,9)				
P value	0.1037				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	0.04292	2	0.02146	F(2,9)=1.740	P=0.2297
Residual (within columns)	0.1110	9	0.01233		
Total	0.1539	11			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	12				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July 1-4 Moon vs. Heat Stress	0.1240	-0.09526 to 0.3432	No	ns	0.3029
July 1-4 Moon vs. Post Heat Stress	0.1296	-0.08967 to 0.3488	No	ns	0.2754
Heat Stress vs. Post Heat Stress	0.005583	-0.2137 to 0.2248	No	ns	0.9972
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July 1-4 Moon vs. Heat Stress	0.1296	0.005583	0.1240	0.07852	
July 1-4 Moon vs. Post Heat Stress	0.1296	0.000	0.1296	0.07852	
Heat Stress vs. Post Heat Stress	0.005583	0.000	0.005583	0.07852	

Supplements 28. One-way ANOVA test and Tukey's multiple comparisons test for superoxide dismutase activity values over July and August reproductive timepoints.

ANOVA summary					
F	4.871				
P value	0.0005				
P value summary	***				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.4602				
Brown-Forsythe test					
F (DFn, DFd)	0.7381(7,40)				
P value	0.6411				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	8.418				
P value	0.2972				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	10817	7	1545	F(7,40)=4.871	P=0.0005
Residual (within columns)	12690	40	317.3		
Total	23508	47			
Data summary					
Number of treatments (columns)	8				
Number of values (total)	48				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
July New Moon vs. July 1/4 Moon	11	-21.87 to 43.87	No	ns	0.9594
July New Moon vs. July Full Moon	53.29	20.42 to 86.16	Yes	***	0.0002
July New Moon vs. July 3/4 Moon	20.01	-12.86 to 52.88	No	ns	0.5292
July New Moon vs. Aug New Moon	16.17	-16.71 to 49.04	No	ns	0.7635
July New Moon vs. Aug 1/4 Moon	5.771	-27.1 to 38.64	No	ns	0.9991
July New Moon vs. Aug Full Moon	17.64	-15.23 to 50.51	No	ns	0.6777
July New Moon vs. Aug 3/4 Moon	12.03	-20.84 to 44.9	No	ns	0.9359
July 1/4 Moon vs. July Full Moon	42.29	9.416 to 75.16	Yes	**	0.0043
July 1/4 Moon vs. July 3/4 Moon	9.008	-23.86 to 41.88	No	ns	0.9866
July 1/4 Moon vs. Aug New Moon	5.163	-27.71 to 38.03	No	ns	0.9996
July 1/4 Moon vs. Aug 1/4 Moon	-5.232	-38.1 to 27.64	No	ns	0.9996

July 1/4 Moon vs. Aug Full Moon	6.634	-26.24 to 39.51	No	ns	0.9979
July 1/4 Moon vs. Aug 3/4 Moon	1.025	-31.85 to 33.9	No	ns	>0.9999
July Full Moon vs. July 3/4 Moon	-33.28	-66.15 to -0.408	Yes	*	0.0454
July Full Moon vs. Aug New Moon	-37.12	-70 to -4.253	Yes	*	0.0174
July Full Moon vs. Aug 1/4 Moon	-47.52	-80.39 to -14.65	Yes	***	0.0009
July Full Moon vs. Aug Full Moon	-35.65	-68.52 to -2.781	Yes	*	0.0253
July Full Moon vs. Aug 3/4 Moon	-41.26	-74.13 to -8.391	Yes	**	0.0057
July 3/4 Moon vs. Aug New Moon	-3.845	-36.72 to 29.03	No	ns	>0.9999
July 3/4 Moon vs. Aug 1/4 Moon	-14.24	-47.11 to 18.63	No	ns	0.8588
July 3/4 Moon vs. Aug Full Moon	-2.373	-35.24 to 30.5	No	ns	>0.9999
July 3/4 Moon vs. Aug 3/4 Moon	-7.983	-40.85 to 24.89	No	ns	0.9935
Aug New Moon vs. Aug 1/4 Moon	-10.39	-43.27 to 22.48	No	ns	0.9701
Aug New Moon vs. Aug Full Moon	1.472	-31.4 to 34.34	No	ns	>0.9999
Aug New Moon vs. Aug 3/4 Moon	-4.138	-37.01 to 28.73	No	ns	>0.9999
Aug 1/4 Moon vs. Aug Full Moon	11.87	-21.01 to 44.74	No	ns	0.9401
Aug 1/4 Moon vs. Aug 3/4 Moon	6.257	-26.61 to 39.13	No	ns	0.9986
Aug Full Moon vs. Aug 3/4 Moon	-5.609	-38.48 to 27.26	No	ns	0.9993

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July New Moon vs. July 1/4 Moon	156.2	145.2	11	10.28	
July New Moon vs. July Full Moon	156.2	102.9	53.29	10.28	
July New Moon vs. July 3/4 Moon	156.2	136.2	20.01	10.28	
July New Moon vs. Aug New Moon	156.2	140	16.17	10.28	
July New Moon vs. Aug 1/4 Moon	156.2	150.4	5.771	10.28	
July New Moon vs. Aug Full Moon	156.2	138.6	17.64	10.28	
July New Moon vs. Aug 3/4 Moon	156.2	144.2	12.03	10.28	
July 1/4 Moon vs. July Full Moon	145.2	102.9	42.29	10.28	
July 1/4 Moon vs. July 3/4 Moon	145.2	136.2	9.008	10.28	
July 1/4 Moon vs. Aug New Moon	145.2	140	5.163	10.28	
July 1/4 Moon vs. Aug 1/4 Moon	145.2	150.4	-5.232	10.28	
July 1/4 Moon vs. Aug Full Moon	145.2	138.6	6.634	10.28	
July 1/4 Moon vs. Aug 3/4 Moon	145.2	144.2	1.025	10.28	
July Full Moon vs. July 3/4 Moon	102.9	136.2	-33.28	10.28	
July Full Moon vs. Aug New Moon	102.9	140	-37.12	10.28	
July Full Moon vs. Aug 1/4 Moon	102.9	150.4	-47.52	10.28	
July Full Moon vs. Aug Full Moon	102.9	138.6	-35.65	10.28	

July Full Moon vs. Aug 3/4 Moon	102.9	144.2	-41.26	10.28	
July 3/4 Moon vs. Aug New Moon	136.2	140	-3.845	10.28	
July 3/4 Moon vs. Aug 1/4 Moon	136.2	150.4	-14.24	10.28	
July 3/4 Moon vs. Aug Full Moon	136.2	138.6	-2.373	10.28	
July 3/4 Moon vs. Aug 3/4 Moon	136.2	144.2	-7.983	10.28	
Aug New Moon vs. Aug 1/4 Moon	140	150.4	-10.39	10.28	
Aug New Moon vs. Aug Full Moon	140	138.6	1.472	10.28	
Aug New Moon vs. Aug 3/4 Moon	140	144.2	-4.138	10.28	
Aug 1/4 Moon vs. Aug Full Moon	150.4	138.6	11.87	10.28	
Aug 1/4 Moon vs. Aug 3/4 Moon	150.4	144.2	6.257	10.28	
Aug Full Moon vs. Aug 3/4 Moon	138.6	144.2	-5.609	10.28	

Supplements 29. One-way ANOVA test and Tukey's multiple comparisons test for superoxide dismutase activity values over acute reproductive timepoints on and consecutive four days following the August ¼ moon reproductive peak.

ANOVA summary					
F	6.608				
P value	0.0009				
P value summary	***				
Sig. diff. among means (P < 0.05)?	Yes				
R square	0.5139				
Brown-Forsythe test					
F (DFn, DFd)	1.078(4,25)				
P value	0.3882				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	4.864				
P value	0.3016				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	5447	4	1362	F(4,25)=6.608	P=0.0009
Residual (within columns)	5152	25	206.1		
Total	10599	29			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	30				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
day 1 vs. day 2	5.313	-19.03 to 29.65	No	ns	0.9667
day 1 vs. day 3	-6.904	-31.24 to 17.44	No	ns	0.9178
day 1 vs. day 4	-22.01	-46.35 to 2.334	No	ns	0.0903
day 1 vs. day 5	18.77	-5.571 to 43.11	No	ns	0.1897
day 2 vs. day 3	-12.22	-36.56 to 12.12	No	ns	0.5879
day 2 vs. day 4	-27.32	-51.66 to	Yes	*	0.0224

		-2.979			
day 2 vs. day 5	13.46	-10.88 to 37.8	No	ns	0.4968
day 3 vs. day 4	-15.1	-39.44 to 9.238	No	ns	0.3837
day 3 vs. day 5	25.67	1.333 to 50.02	Yes	*	0.0351
day 4 vs. day 5	40.78	16.44 to 65.12	Yes	***	0.0004
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
day 1 vs. day 2	150.4	145.1	5.313	8.288	
day 1 vs. day 3	150.4	157.3	-6.904	8.288	
day 1 vs. day 4	150.4	172.4	-22.01	8.288	
day 1 vs. day 5	150.4	131.7	18.77	8.288	
day 2 vs. day 3	145.1	157.3	-12.22	8.288	
day 2 vs. day 4	145.1	172.4	-27.32	8.288	
day 2 vs. day 5	145.1	131.7	13.46	8.288	
day 3 vs. day 4	157.3	172.4	-15.1	8.288	
day 3 vs. day 5	157.3	131.7	25.67	8.288	
day 4 vs. day 5	172.4	131.7	40.78	8.288	

Supplements 30. One-way ANOVA test and Tukey's multiple comparisons test for superoxide dismutase activity values over pre-bleaching, bleaching, and post-bleaching timepoints.

ANOVA summary					
F	3.297				
P value	0.0843				
P value summary	ns				
Sig. diff. among means (P < 0.05)?	No				
R square	0.4228				
Brown-Forsythe test					
F (DFn, DFd)	0.3347(2,9)				
P value	0.7241				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	0.2280				
P value	0.8922				
P value summary	ns				
SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F(DFn,DFd)	P value
Treatment (between columns)	1060	2	529.9	F(2,9)=3.297	P=0.0843
Residual (within columns)	1447	9	160.7		
Total	2506	11			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	12				

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
July 1-4 Moon vs. Heat Stress	23.02	-2.012 to 48.05	No	ns	0.0709
July 1-4 Moon vs. Post Heat Stress	11.73	-13.30 to 36.76	No	ns	0.4256
Heat Stress vs. Post Heat Stress	-11.29	-36.32 to 13.74	No	ns	0.4511

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	
July 1-4 Moon vs. Heat Stress	146.2	123.2	23.02	8.965	
July 1-4 Moon vs. Post Heat Stress	146.2	134.5	11.73	8.965	
Heat Stress vs. Post Heat Stress	123.2	134.5	-11.29	8.965	

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